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13. Abstract *(Maximum 200 Words)* *(abstract should contain no proprietary or confidential information)*

The Brn-3b POU family transcription factor is over-expressed in breast cancer cells compared to normal mammary cells. In this project, we have stably transfected the human breast cancer cell line MCF-7 with vectors expressing the sense or anti-sense strand of Brn-3b so as to isolate cell lines with respectively enhanced or reduced Brn-3b levels. The cell lines with enhanced Brn-3b levels show enhanced growth rate, saturation density, cell division, anchorage independence *in vitro* and enhanced ability to form tumors *in vivo*. In contrast, the cell lines with reduced Brn-3b show reductions in all those growth parameters. In addition, the cell lines with altered Brn-3b levels show altered expression of genes such as BRCA-1 and the oestrogen receptor whose expression is known to be modulated in breast cancer. These findings thus establish Brn-3b as a key factor regulating gene expression and growth of human breast cancer cells.

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Table of Contents

Regulation of BRCA-1 Gene Expression and Mammary Tumorigenesis by the Brn-3b POU Family Transcription Factor

Grant Holder: Professor D.S. Latchman

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Introduction

This project is based on our finding that the Brn-3b POU family transcription factor is over-expressed in breast cancer cells, compared to its expression in normal mammary epithelial cells. Moreover, prior to the start of the project, we demonstrated that over-expression of Brn-3b correlated with reduced expression of the BRCA-1 anti-oncogene in the breast tumour cells (1). The aim of this project was therefore to evaluate the role of Brn-3b in regulating the general growth and gene expression pattern of breast cancer cells and its role in particular, in regulating BRCA-1 gene expression.

Body

The project had two specific tasks. The research accomplishments associated with each task are described below.

Task 1: Analysis of the mechanism by which Brn-3b inhibits the BRCA-1 promoter

In our previous experiments we demonstrated that Brn-3b strongly represses the activity of the BRCA-1 promoter in co-transfection assays (1). This provides a mechanism for the inverse correlation between Brn-3b and BRCA-1 gene expression in mammary tumour cells (see above) and suggested that Brn-3b may play an important role in regulating BRCA-1 gene expression in mammary cells.

To further characterise the effect of Brn-3b on the BRCA-1 gene promoter, we used progressively shorter BRCA-1 promoter constructs in co-transfection experiments. By this means we demonstrated that the repression of the BRCA-1 promoter by Brn-3b could be observed in co-transfection experiments with a construct containing only 500 bases of the BRCA-1 promoter, significantly extending our earlier observations which utilised a construct containing 3,500 bases of the promoter.

However, despite its small size, this short promoter fragment contains two distinct promoters, the α and β promoters which can both independently drive BRCA-1 gene expression (2). We therefore tested constructs containing either the isolated α or the isolated β promoter in co-transfection experiments. In these experiments (see Appendix Fig.1) both the construct containing the isolated α promoter and the construct containing the isolated β promoter were strongly repressed by Brn-3b. Hence, repression by Brn-3b is a property shared by these two distinct BRCA-1 promoters.

In our previous experiments, we had also tested the effect of the Brn-3a transcription factor which is closely related to Brn-3b. We found that the Brn-3a factor was also able to repress the large BRCA-1 promoter fragment and this effect was noted for the isolated α and β promoters also (see Appendix Fig.1). However, these experiments involved the short form of

Brn-3a and did not utilise the long form of Brn-3a which contains an additional 84 amino acids. In experiments where we tested the effect of the long form of Brn-3a for the first time, we were able to show that it was able to repress the isolated BRCA-1 β promoter but was able to activate the BRCA-1 α promoter (see Appendix Fig.1).

These experiments therefore show for the first time that a specific form of a Brn-3 factor can activate a BRCA-1 promoter and moreover, that the two different BRCA-1 promoters respond differently to Brn-3a. These experiments involving a differential effect of the two different forms of Brn-3a are of particular interest since both these forms occur naturally and the alternative splicing event which generates them is regulated in different cell types and in response to different stimuli (3).

The experiments carried out in this project have therefore defined a short region which is necessary for the response to Brn-3b and the closely related factor Brn-3a. Moreover, they have shown that Brn-3b and the short form of Brn-3a can repress both the α and β promoters, whereas the long form of Brn-3a can activate the α promoter whilst repressing the β promoter. These results therefore represent a considerable advance over our initial observation (1) that a large fragment of the BRCA-1 promoter was repressed by Brn-3b.

Task 2: Characterisation of the effects of manipulating Brn-3b expression on the expression of BRCA-1 and the proliferation of normal and malignant mammary cells

In view of the over-expression of Brn-3b in breast cancer cells compared to normal mammary epithelium, we wished to observe the effects of manipulating Brn-3b expression in mammary cells on their growth rate and pattern of gene expression. To do this, the human breast cancer cell line MCF7 was transfected either with an expression vector capable of directing the over-expression of Brn-3b or with a vector containing the antisense strand of Brn-3b which should reduce the endogenous Brn-3b expression. Parallel samples of cells were also transfected with empty expression vector. Following transfection, stably transfected cells were isolated and cell lines grown.

In these experiments, we identified two cell lines of each type which demonstrated the appropriate change in Brn-3b expression. Thus, cells transfected with the expression vector for Brn-3b showed approximately ten-fold over-expression of Brn-3b compared to control cells. Conversely, cells transfected with the antisense Brn-3b construct showed abolished Brn-3b expression (see Appendix Fig.2).

In view of the potential importance of these cell lines in analysing the manner in which Brn-3b expression contributes to mammary tumorigenesis and the supportive comments on this aspect of the work obtained in reviews of our previous reports, we have devoted our major attention to the characterisation of these cell lines.

In these experiments, the cell lines over-expressing Brn-3b showed both enhanced population doubling times as well as enhanced cellular density at the plateau phase compared to cells transfected with empty expression vector. Conversely, the cells with reduced Brn-3 levels due to transfection with the antisense plasmids showed a reduced growth rate and cellular density at plateau phase compared to the control cells (see Appendix, Figs.3 and 4). These results were confirmed by measuring the rate of cell division in each of the cell types by means of tritiated thymidine incorporation which demonstrated enhanced cell division in the cells over-expressing Brn-3b and reduced cell division in the cells with lower Brn-3b levels (see Appendix, Fig.5).

These results demonstrate therefore, that over-expression of Brn-3b (as occurs in mammary tumours) results in enhanced growth rate of the MCF7 cells, whereas reduced growth rate is observed in the antisense cells having reduced Brn-3b levels.

In view of our previous observation that Brn-3b interacts with the oestrogen receptor and modulates its activity (4), we tested whether altered expression of Brn-3b also modulated the oestrogen responsiveness of MCF-7 cells. In these experiments (see Appendix, Fig.6) we observed the same differences in growth rate between the various cell lines independent of whether oestrogen was present. Hence, Brn-3b can modulate the growth rate of MCF-7 cells in the presence or absence of oestrogen. However, the strongest response to oestrogen was observed in the Brn-3b over-expressing cells in stripped serum lacking any endogenous oestrogen. Hence, as well as enhancing the growth rate of MCF-7 cells, Brn-3b also enhances their response to oestrogen. This is in agreement with our previous finding that Brn-3b interacts directly with the oestrogen receptor and enhances its ability to activate its target gene promoters (4).

In experiments with the oestrogen antagonist tamoxifen (see Appendix, Fig.6), all the cell lines showed decreased growth, as expected. Importantly however, the differences in growth rate of the various cell lines were still maintained under these conditions. Hence, Brn-3b both enhances the oestrogen responsiveness of cellular growth rate and stimulates cellular growth independently of oestrogen.

These observations were further extended by measuring the ability of the various cells to form colonies in soft agar. This is a measure of their ability to grow in an anchorage-independent manner which is predictive of tumour growth *in vivo*. In these experiments (see Appendix, Fig.7) the Brn-3b over-expressing cell line (Z) showed statistically significantly enhanced ability to form colonies compared to the control cells. Most importantly, the antisense cell clone (A1) showed a very dramatically reduced ability to form colonies in soft agar. Hence, anchorage independence is dramatically reduced in the absence of high level expression of Brn-3b, indicating that the elevated levels of Brn-3b observed in mammary tumours cells may play a key role in their tumorigenic behaviour.

As anchorage-independent growth is a predictor of the ability of tumour cells to grow *in vivo*, we inoculated our tumour cells into nude mice. This work clearly demonstrated that the over-expression of Brn-3b in MCF7 cells dramatically enhanced their ability to grow and form tumours in nude mice. Hence, enhanced Brn-3b levels not only enhance the ability to grow *in vitro* and the ability to grow in an anchorage-independent manner in culture but also enhance tumour growth *in vivo*.

In view of the clear effects of altered expression of Brn-3b on tumour cell growth both *in vitro* and *in vivo*, we wished to examine the molecular mechanism of this effect. Thus, Brn-3b is a transcription factor and is likely to act by altering the expression of specific target genes. This analysis was regarded by the Grants Officer as an extension to Task 2, not requiring any change in the POW.

In these experiments, we first examined the expression of a number of different genes which are of potential importance in human breast cancer. Expression was analysed both at the mRNA level by reverse transcriptase/polymerase chain reaction (RT-PCR) and then at the protein level by western blotting. As expected, enhanced expression of BRCA-1 was observed in the Brn-3b antisense cell lines, whereas reduced expression of BRCA-1 was observed in the Brn-3b over-expressing cell lines. In further experiments we showed that Brn-3b over-expressing cells, in contrast to their reduced expression of BRCA-1 showed enhanced

expression of the oestrogen receptor (with which Brn-3b interacts: 4), human chorionic gonadotrophin and of the 27 kDa heat shock protein HSP27 all of which have been observed to be over-expressed in human breast cancer. As a control, we observed no change in the expression of other heat shock proteins HSP70 and HSP90. A typical result of this experiment is illustrated in Fig.8.

These experiments therefore extend our initial observation of regulation of BRCA-1 by Brn-3b to show that the expression of several different genes known to be altered in human breast cancer is also altered in the cells with altered Brn-3b expression. To follow this up further, we wished to use a global analysis of gene expression in order to isolate novel genes whose expression was altered by changing Brn-3b levels in mammary cells, without making any *a priori* assumptions about their nature.

We therefore used labelled cDNA prepared from mRNA of the Brn-3b over-expressing or under-expressing cells to probe the Atlas human cancer cDNA array which contains sequences from 1176 cancer-related genes arrayed on a filter. The results of this experiment (Table 1) showed clear alteration in the expression of 51 genes whose expression was either up or down-regulated in the Brn-3b over-expressing cells compared to the under-expressing cells. These potential target genes included a number whose protein products are know to have a role in cellular growth and differentiation and hence their identification as potential target genes for Brn-3b in mammary cells was of great interest.

Accordingly therefore, we have further analysed these genes in two ways. Firstly, we have characterised their expression by real time RT-PCR and western blot analysis in our cell lines having respectively elevated, normal or reduced levels of Brn-3b. This experiment was intended to confirm the results of the initial filter screening assay. Secondly, to establish the relevance of our results for human breast cancer, we have measured the levels of Brn-3b and of selected potential target genes in biopsy samples from human mammary tumours.

In the real time RT-PCR experiments (Appendix, Fig.9) we obtained results which confirmed the filter screening. Thus, we showed, for example, that levels of cyclin-dependent kinase 4 (cdk4), p21 and RhoC were enhanced in the Brn-3b over-expressing cells (Z) compared to the antisense cells (A). Conversely, expression of c-*myc*, fuse binding protein, c-*jun*, EGR-1 and zyxin was reduced in the over-expressing cells (Z) compared to the antisense cells (A). These results therefore, confirm the results of the initial filter screen and show that the expression of several oncogenes and of other genes involved in growth control such as cdk4, can be modulated by Brn-3b.

Most interestingly, when we measured the expression of several of these genes in human breast cancer samples (Appendix, Fig.10), they showed a clear correlation with expression of Brn-3b. This was particularly marked for cdk4 which showed a clear positive correlation with Brn-3b. This contrasted with the expected negative correlation for BRCA-1. Thus, genes isolated as potential targets in our gene array experiments may indeed be regulated by Brn-3b in human breast cancer.

Hence, the global screening experiments have indicated that Brn-3b regulates a number of genes which may have critical roles in regulating the growth and behaviour of mammary cells. Taken together with our other data therefore, these findings indicate that Brn-3b is likely to be a key regulator of mammary tumour growth acting via the up or down regulation of cellular genes involved in this process.

Key research accomplishments

- Identified a short region of the BRCA-1 promoter which is necessary and sufficient to mediate its response to Brn-3b. Demonstrated that both the α and β BRCA-1 gene promoters are independently regulated by Brn-3b.

- Demonstrated that the long form of the related POU family transcription factor Brn-3a can activate the α but not the β BRCA-1 promoter, whereas the short form of Brn-3a or Brn-3b repress both these promoters.

- Isolated MCF7 human breast cancer-derived cell lines with enhanced or reduced levels of Brn-3b, compared to parental cells.

- Demonstrated that cells with enhanced Brn-3b levels show enhanced growth rates, saturation density and cell division, whereas cells with reduced Brn-3b levels show reduced growth rates, saturation density and cell division, thereby demonstrating for the first time the critical role of Brn-3b in regulating breast cancer cell growth *in vitro*.

- Demonstrated that cells over-expressing Brn-3b show enhanced anchorage-independent growth and growth *in vivo* to form tumours, whereas the opposite is true for the Brn-3b antisense cells, demonstrating the importance of Brn-3b for tumour growth *in vivo*.

- Demonstrated that the expression of specific genes such as BRCA-1 oestrogen receptor chorionic gonadotrophin and HSP27 is altered in the cells with altered Brn-3b expression. Used global analysis of gene expression to identify novel target genes whose expression is increased or decreased in response to altered Brn-3b expression.

- Demonstrated that in several genes identified in the global analysis, such as cdk4, expression of the gene correlates with that of Brn-3b in human breast cancer samples.

Reportable outcomes

- A paper reporting the effect of manipulating Brn-3b expression on the growth rate of MCF7 cells has been published (5).

- A further paper reporting the results of the global gene expression analysis of these cells is currently be prepared.

- MCF7 cells over-expressing Brn-3b or exhibiting reduced Brn-3b levels have now been extensively characterised and are available for use by other investigators.

- A number of genes whose expression is affected by altered Brn-3b levels in the MCF7 cells have now been identified both by testing individual genes, known to show altered expression in breast cancer and by a global gene expression analysis which has identified novel potential targets for Brn-3b.

Conclusions

The major achievement of this project is the demonstration that over-expression of Brn-3b in human mammary tumour cells enhances their growth rate and ability to form tumours, as well as their oestrogen responsiveness, whereas a corresponding reduction in Brn-3b reduces this.

This establishes Brn-3b as a potential regulator of mammary tumour growth and when taken together with our earlier observation of its over-expression in human breast cancer (1) establishes it as a key regulator and potential therapeutic target in human breast cancer.

As a transcription factor, Brn-3b is likely to act by regulating the expression of other target genes either positively or negatively. In agreement with this idea, we have demonstrated that several genes which are known to have altered expression in breast cancer, also show altered expression in the cell lines with altered Brn-3b expression. These include the BRCA-1 gene which we previously demonstrated showed an inverse correlation in expression pattern with Brn-3b in human tumour samples (1). By further analysis of the BRCA-1 promoter, we have defined the short regions of the two promoters which mediate their response to Brn-3b. In addition, by using global gene expression analysis, we have identified novel target genes which are induced or repressed by altering Brn-3b expression and several of which are known to modulate cellular growth. In some cases such as cdk4, we have shown an appropriate correlation between Brn-3b expression and the expression of these target genes in human breast cancers.

Overall therefore, this project has demonstrated that manipulating Brn-3b in human breast cancer cells can alter their growth characteristics and the expression of specific genes and that moreover, the over-expression of Brn-3b in human breast cancers is paralleled by the altered expression of its target genes, Brn-3b is likely therefore, to play a significant role in the tumorigenic process in human breast cancer and may be a potential therapeutic target.

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* Publication arising from the project.

Appendix

Table I. Results of the differential display of genes on the Atlas human cancer cDNA array

experimental		vs.	control
brn-3b short			anti-sense

Normalization		Thresholds	
method	coefficient	ratio	difference
global (sum)	1.30	1.69	15

Array lot#
9100044
Atlas Array
Cancer 1.2k Array

#	coordinate	Spot Intensity anti-sense	Spot Intensity brn-3b short	Ratio	Difference	RATIO UP	RATIO DOWN	? = weak signal (low trust) Gene
1	A01c	66	28	0.42	-38		2.4	c-jun proto-oncogene; transcription factor AP-1
2	A01h	3	25	8.33	22	8.3		interferon-inducible protein 9-27
3	A03c	71	36	0.51	-35		2.0	c-myc oncogene
4	A03g	256	533	2.08	277	2.1		c-myc binding protein MM-1
5	A04j	51	88	1.73	37	1.7		cell division protein kinase 4; cyclin-dependent kinase 4 (CDK4); PSK-J3
6	A09l	50	116	2.32	66	2.3		cyclin-dependent kinase inhibitor 1 (CDKN1A); melanoma differentiation-associated protein 6 (MDA6); CDK-interacting protein 1 (CIP1); WAF1
7	A10k	84	142	1.69	58	1.7		cyclin-dependent kinase regulatory subunit 1 (CKS1)
8	A12j	44	24	0.55	-20		1.8	cdc2-related protein kinase PISSLRE
9	A12n	35	10	0.29	-25		3.5	G1 to S phase transition protein 1 homolog; GTP-binding protein GST1-HS
10	B02a	381	220	0.58	-161		1.7	ADP/ATP carrier protein
11	B02i	49	23	0.47	-26		2.1	protein phosphatase 2C gamma
12	B04j	68	129	1.90	61	1.9		rhoC (H9); small GTPase (rhoC)
13	B04n	170	288	1.69	118	1.7		B-cell receptor-associated protein (hBAP)
14	B05l	35	18	0.51	-17		1.9	? calmodulin 1; delta phosphorylase kinase
15	B07m	41	3	0.07	-38		13.7	zyxin + zyxin-2
16	B10c	30	51	1.70	21	1.7		c-jun N-terminal kinase 2 (JNK2); JNK55
17	B10m	167	37	0.22	-130		4.5	junction plakoglobin (JUP); desmoplakin III (DP3)
18	B13j	29	10	0.34	-19		2.9	? guanine nucleotide-binding protein G(I)/G(S)/G(O) gamma-10 subunit
19	C01g	46	19	0.41	-27		2.4	DNA ligase I; polydeoxyribonucleotide synthase (ATP) (DNL1) (LIG1)
20	C04b	127	237	1.87	110	1.9		tumor necrosis factor type 1 receptor associated protein (TRAP1)
21	C04g	34	19	0.56	-15		1.8	? DNA excision repair protein ERCC1
22	C06n	27	10	0.37	-17		2.7	? interferon regulatory factor 3 (IRF3)
23	C08l	27	7	0.26	-20		3.9	? retinoic acid receptor alpha 1 (RAR-alpha 1; RARA) + PML-RAR protein
24	C12i	40	5	0.13	-35		8.0	TIS11B protein; EGF response factor 1 (ERF1)
25	C12j	28	5	0.18	-23		5.6	early growth response protein 1 (hEGR1); transcription factor ETR103; KROX24; zinc finger protein 225; AT225
26	C14m	25	3	0.12	-22		8.3	fuse-binding protein 2 (FBP2)
27	C14n	49	24	0.49	-25		2.0	transcription factor erf-1; AP2 gamma transcription factor
28	D06e	60	32	0.53	-28		1.9	integrin beta 4 (ITGB4); CD104 antigen
29	D07b	65	31	0.48	-34		2.1	high mobility group protein HMG2
30	D08f	37	11	0.30	-26		3.4	paxillin
31	D09d	44	19	0.43	-25		2.3	alpha1 catenin (CTNNA1); cadherin-associated protein; alpha E-catenin
32	D09m	55	109	1.98	54	2.0		glutathione-S-transferase (GST) homolog
33	D14i	19	3	0.16	-16		6.3	? cysteine-rich fibroblast growth factor receptor ; Golgi membrane sialoglycoprotein MG160 (GLG1)
34	E02n	55	10	0.18	-45		5.5	78-kDa glucose regulated protein precursor (GRP 78); immunoglobulin heavy chain binding protein (BIP)
35	E03j	780	384	0.49	-396		2.0	cathepsin D precursor (CTSD)
36	E07f	130	64	0.49	-66		2.0	interleukin-1 beta precursor (IL-1 ; IL1B); catabolin
37	E07h	239	440	1.84	201	1.8		macrophage migration inhibitory factor (MIF); glycosylation-inhibiting factor (GIF)
38	E09h	25	9	0.36	-16		2.8	? jagged2 (JAG2)
39	F04k	61	29	0.48	-32		2.1	60S ribosomal protein L5
40	F05e	14	36	2.57	22	2.6		ornithine decarboxylase
41	F08j	30	15	0.50	-15		2.0	? HSC70-interacting protein; progesterone receptor-associated P48 protein
42	F08k	28	9	0.32	-19		3.1	? eukaryotic translation initiation factor 3 beta subunit (EIF-3 beta); EIF3 P116
43	F08m	75	19	0.25	-56		3.9	PM5 protein
44	F10b	24	9	0.38	-15		2.7	? IMP dehydrogenase 1
45	F10j	110	64	0.58	-46		1.7	suppressor for yeast mutant
46	F12d	25	10	0.40	-15		2.5	? uridine 5'-monophosphate synthase (UMP synthase)
47	F12f	141	77	0.55	-64		1.8	type II cytoskeletal 2 epidermal keratin (KRT2E); cytokeratin 2E (K2E; CK2E)
48	F13k	52	15	0.29	-37		3.5	glycyl tRNA synthetase
49	F14b	24	42	1.75	18	1.8		aminoacylase 1 (ACY1)
50	G27	2180	5230	2.40	3050	2.4		liver glyceraldehyde 3-phosphate dehydrogenase (GAPDH)
51	G43	1004	397	0.40	-607		2.5	cytoplasmic beta-actin (ACTB)

N/C = not calculated due to manually-determined inconsistencies (signal bleeding, background, etc.) in one or both spots

APPENDIX - FIGURES

Figure 1

Regulation of the BRCA-1 α and β promoters by Brn-3 factors in MCF-7 cells. Results of co-transfecting constructs in which the BRCA-1 α (Panel A) or BRCA-1β (Panel B) promoters drive the expression of a luciferase reporter gene. The results are shown for co-transfections involving expression vector lacking any insert (LTR) or expressing Brn-3a long form, Brn-3a short form or Brn-3b.

Figure 2

Levels of Brn-3b in cell lines transfected with the indicated plasmid and assayed by Western blotting using an antibody to Brn-3b. The column headed densitometry shows the results obtained by densitometric scanning in each case with levels equalised for that of the actin control protein, whilst the Table headed fold-expression compares the expression in each cell line to that of control cells transfected with empty expression vector (p LTR).

Figure 3

Growth rate (Panel A) and saturation density limitation (Panel B), studies of MCF-7 stable clones. Two clones over-expressing Brn-3b (Z and Y) control clones (B and C) or anti-sense clones (A1 and A2) were used in each of these experiments. The number of cells at each time point represents the mean of three independent experiments and the error bars indicate the standard deviation of the mean. * Represents statistically differences (p is< 0.05) when over-expressing or anti-sense cell lines were compared to empty vector controls.

Figure 4

Summary of the results illustrated in Fig.3 showing the cell number obtained at the plateau phase in growth curve experiments and the saturation density of each cell line in saturation density limitation experiments.

Figure 5

Tritiated thymidine incorporation by clonal MCF7 cell lines with altered levels of Brn-3b. Cells were grown in full growth medium for 48 hours and subsequently treated with tritiated thymidine. After one hour stimulation, cells were trypsinised, harvested onto glass filters, and counts per minute from the glass filters were recorded by a scintillation counter. The counts per minute from each cell line represent the mean of three independent experiments counted in triplicate +/- the standard deviation of the mean "•" denotes p-value less than 0.05 resulting from a chi-squared test comparing all values at the indicated time point from the experimental (Brn-3b short or Brn-3b antisense) with the expected (pLTR control).

Figure 6

Endocrine characteristics of MCF7 stable clones as measured by cell counts. Two clones representing each of the over-expressing (3bO2), control (V1 and V2), and antisense (3bAS1 and 3bAS2) cell lines were used in each of these experiments. Cells were grown in full growth medium (FGM) or phenol-redless medium containing dextran charcoal-stripped serum (SS) for 48 h and subsequently treated with 17-β estradiol (200 ng/ml), ethanol vehicle, or Tamoxifen (200 ng/ml). Cells were trypsinised and counted on a haemocytometer at the end

of 2 days stimulation. The number of cells for each cell line represents the mean of three independent experiments ± the standard deviation of the mean. (a) Cells grown in full growth medium (FGM), (b) cells grown in phenol-redless medium containing dextran charcoal-stripped serum (SS).

Figure 7

Anchorage independent colony formation by clonal MCF7 cell lines with altered levels of Brn-3b. Cells were plated in full growth 0.3% agarose medium. After 21 days, colonies comprised of at least 32 cells were counted. The number of colonies from each cell line represent the mean of three independent experiments counted in triplicate +/- the standard deviation of the mean. "•" denotes p-value less than 0.05 resulting from a chi-squared test comparing all values at the indicated time point from the experimental (Brn-3b short or Brn-3b antisense) with the expected (pLTR control).

Figure 8

Western blot analysis of beta human chorionic gonadotropin in clonal MCF7 cell lines with altered levels of Brn-3b. Immunoblots to detect the levels of beta-hCG were carried out using six clones: two over-expressing pLTR Brn-3b (Z and Y) each in duplicate, and two empty pLTR vector controls (B and C). Total cellular protein (60 micrograms per lane) was fractionated on a SDS/15% polyacrylamide gel, transferred to nitrocellulose, and probed with an anti-beta-hCG antibody. Expression levels were normalised to total protein densitometry of the Coommassie satined gel after western blotting. The level of beta-hCG antigen in extract from the pLTR over-expressing clones was 5.66 – 14.45 times the average endogenous level seen in the vector controls.

Figure 9

Real time RT-PCR assay measuring the mRNA levels of the indicated genes in MCF-7 cells over-expressing Brn-3b (Z) or with reduced expression (A).

Figure 10

Expression of BRCA-1, p21 and cdk4 in human breast cancer biopsies and its relation to the expression of Brn-3b in the same samples.

Figure 1 (a)

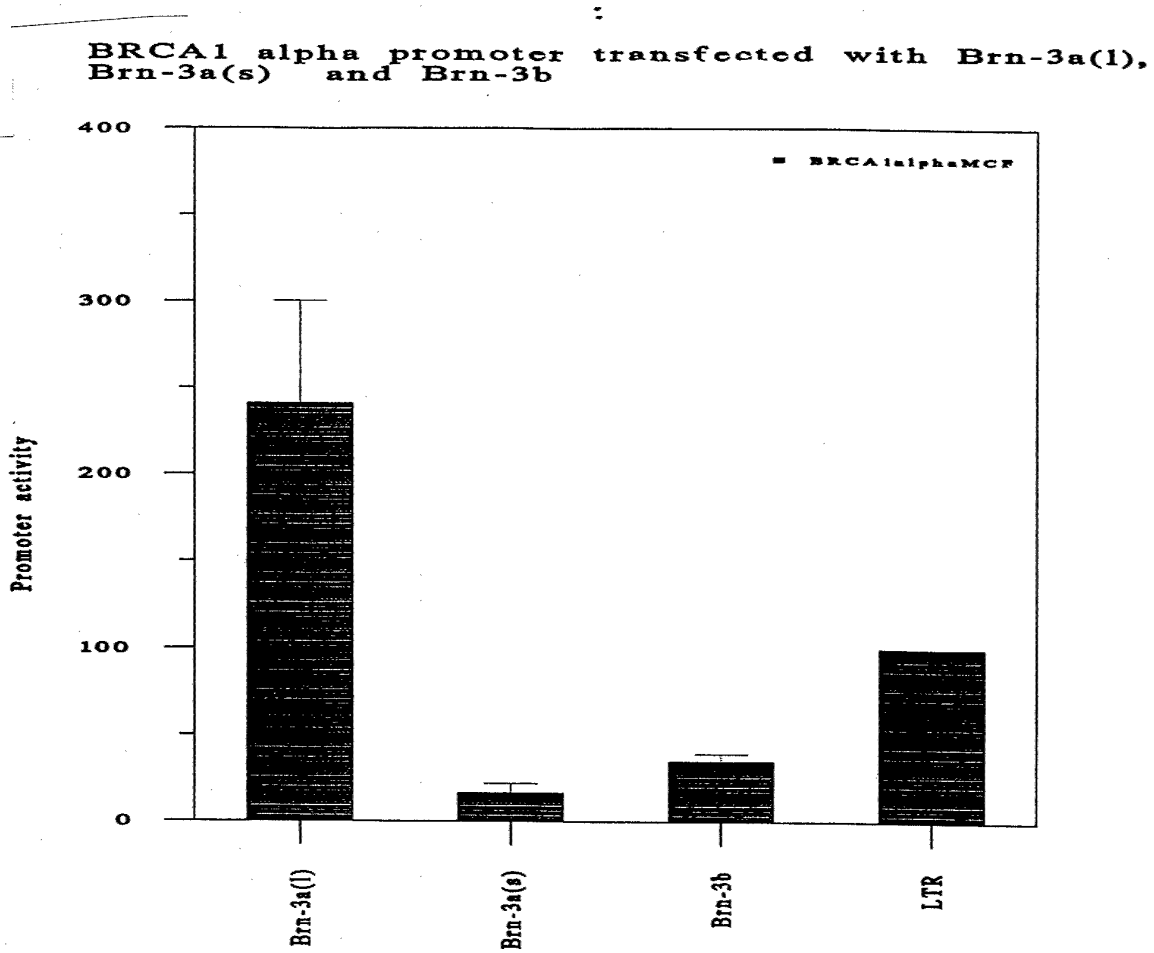


Figure 1 (b)

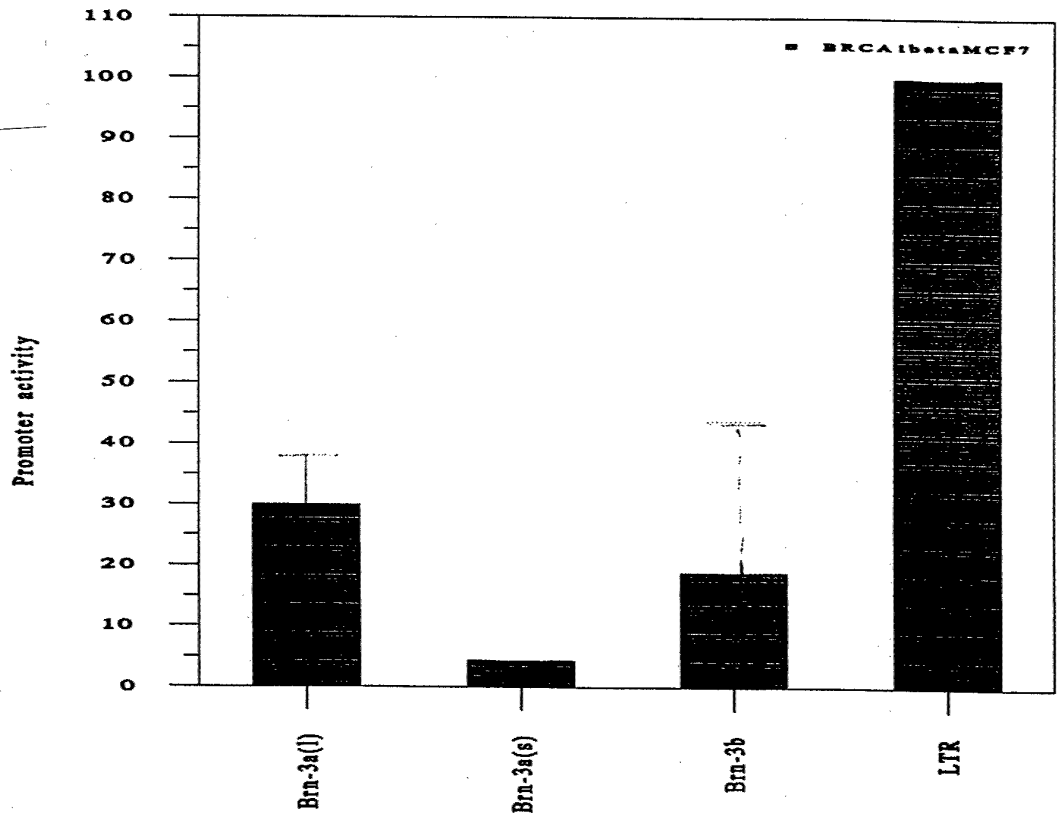
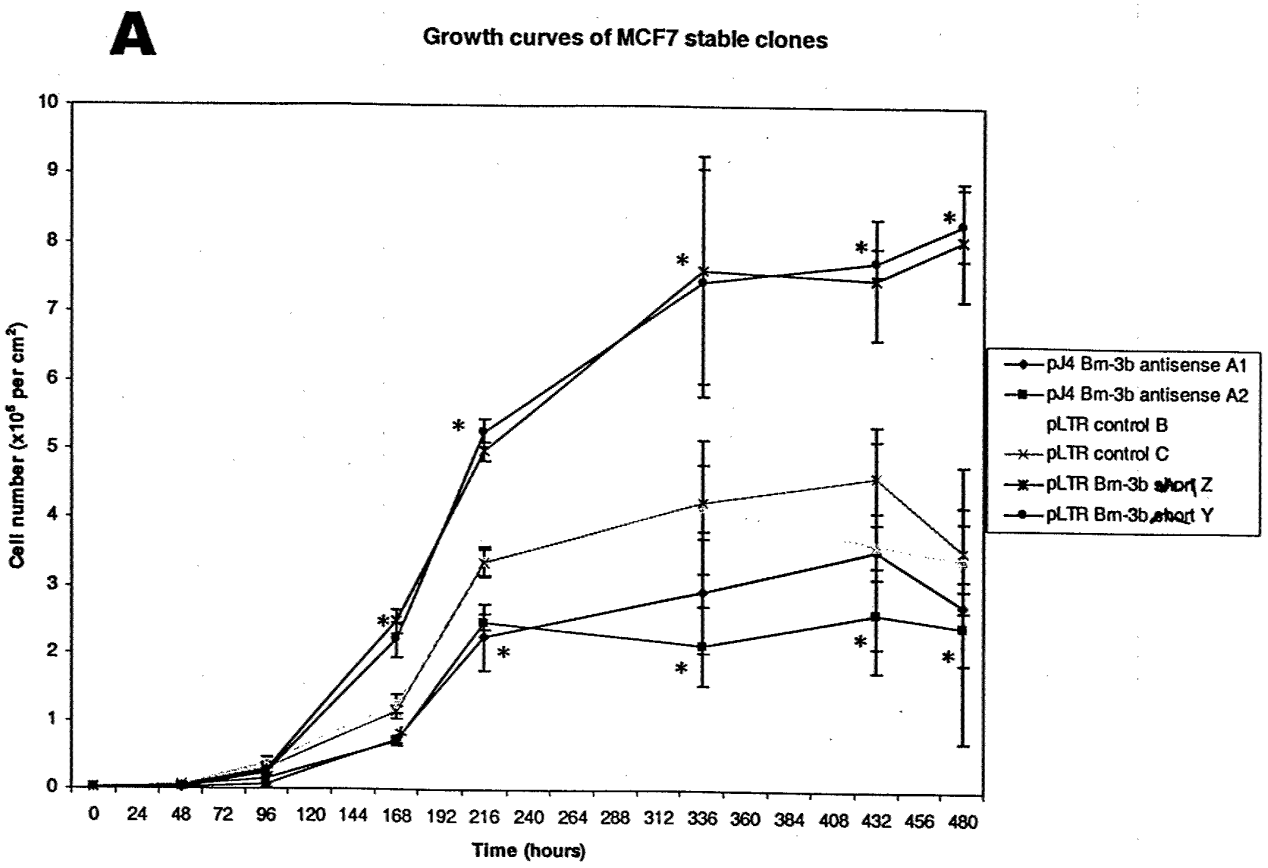


Figure 2

CLONE	DENSITOMETRY (arbitrary units)	FOLD EXPRESSION
	Brn-3b	Brn-3b
pLTR Brn-3b Z	41149	10.6
pLTR Brn-3b Y	45958	11.9
pLTR B	3670	1.0
pLTR C	4072	1.0
pJ4 Brn-3b antisense A1	0	0.0
pJ4 Brn-3b antisense A2	0	0.0

Figure 3


A
Growth curves of MCF7 stable clones
Cell number (x10^5 per cm^2)
Time (hours)
0 24 48 72 96 120 144 168 192 216 240 264 288 312 336 360 384 408 432 456 480
0 1 2 3 4 5 6 7 8 9 10
pJ4 Bm-3b antisense A1
pJ4 Bm-3b antisense A2
pLTR control B
pLTR control C
pLTR Bm-3b short Z
pLTR Bm-3b short Y

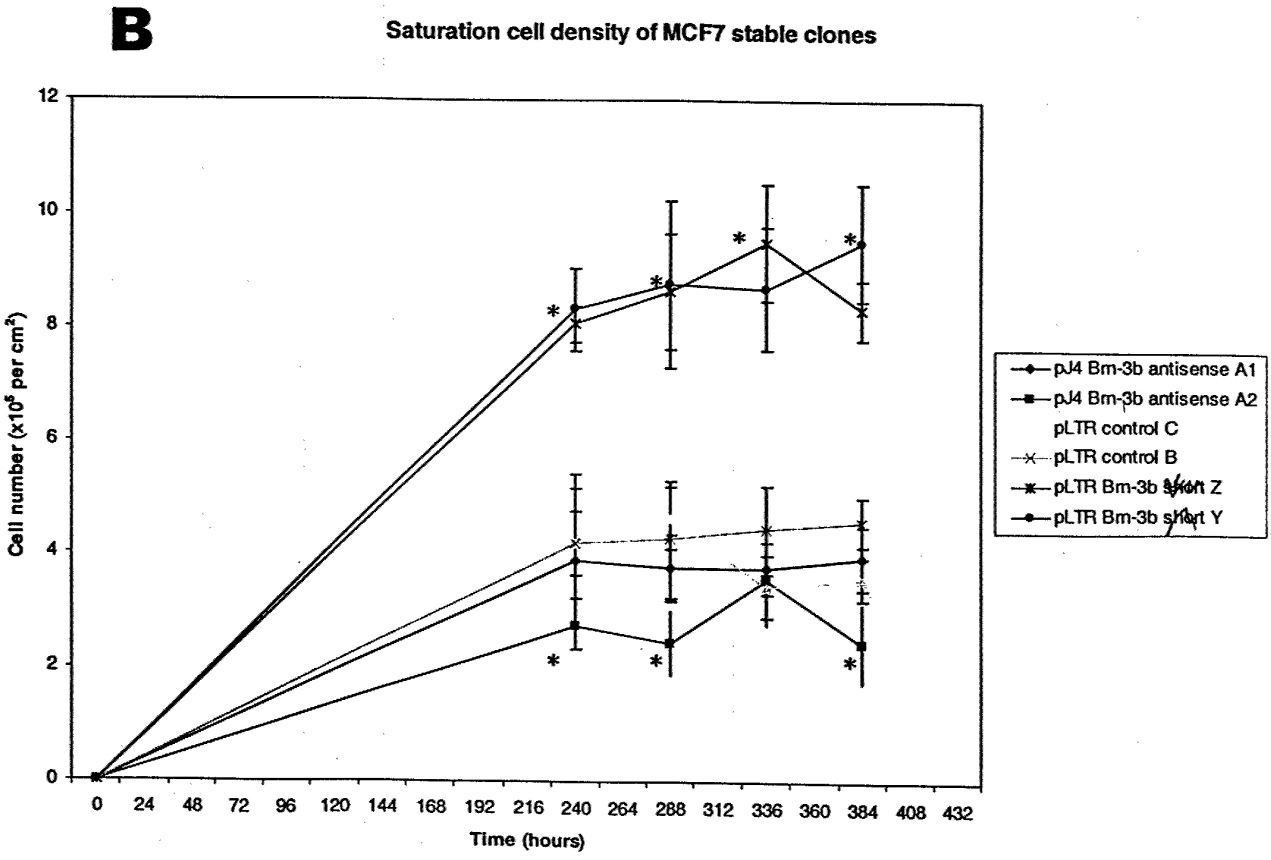

B
Saturation cell density of MCF7 stable clones
Cell number (x10^5 per cm^2)
Time (hours)
0 24 48 72 96 120 144 168 192 216 240 264 288 312 336 360 384 408 432
0 2 4 6 8 10 12
pJ4 Bm-3b antisense A1
pJ4 Bm-3b antisense A2
pLTR control C
pLTR control B
pLTR Bm-3b short Z
pLTR Bm-3b short Y

Figure 4

Growth parameters of MCF-7 stable clones

	Clone	Plateau phase		Saturation Density
		days to	cell number ($x10^5$ per cm^2)	cell number ($x10^5$ per cm^2)
Brn-3b	Z	14	7.66 +/- 0.66	8.63 +/- 0.89
Brn-3b	Y	14	7.43 +/- 0.66	8.81 +/- 1.08
Control	B	12	4.17 +/- 0.39	3.95 +/- 0.82
Control	C	12	4.25 +/- 022	4.31 +/- 0.520
Anti-sense	A1	10	2.92 +/- 0.36	3.83 +/- 0.81
Anti-sense	A2	10	2.12 +/- 0.24	2.64 +/- 0.84

Figure 5

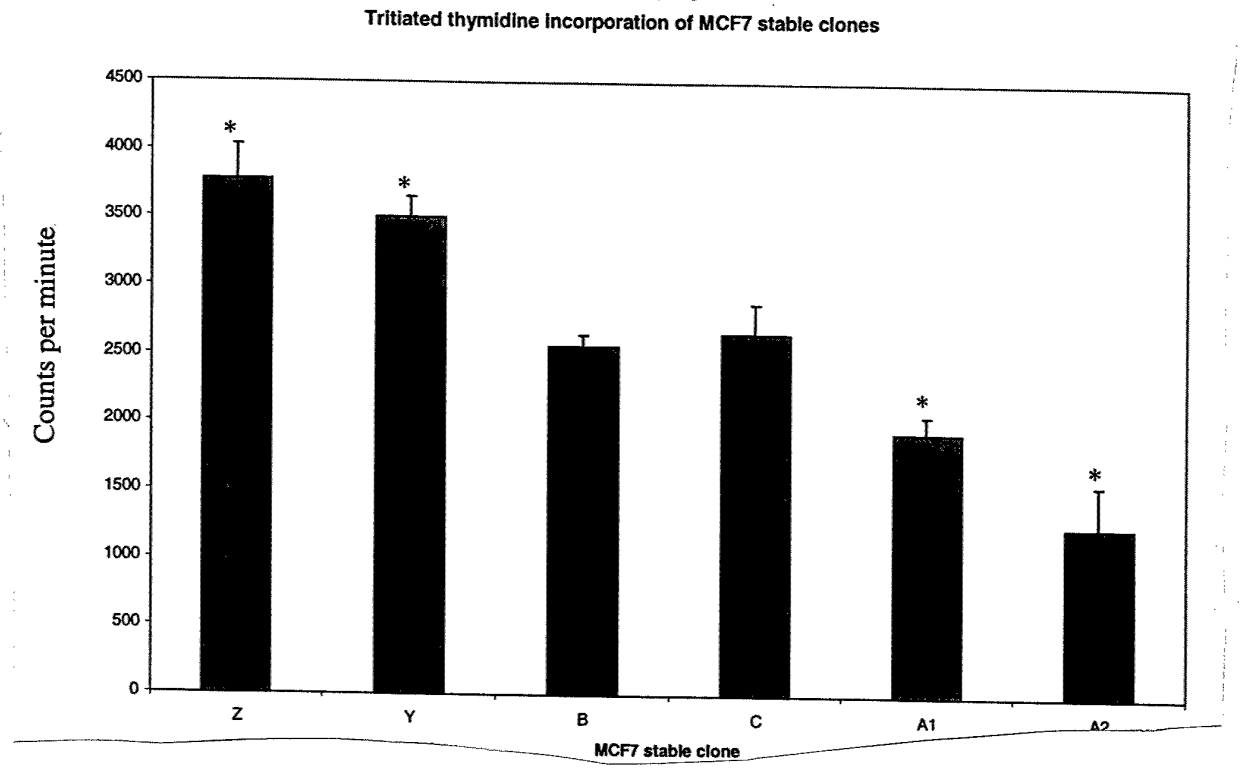
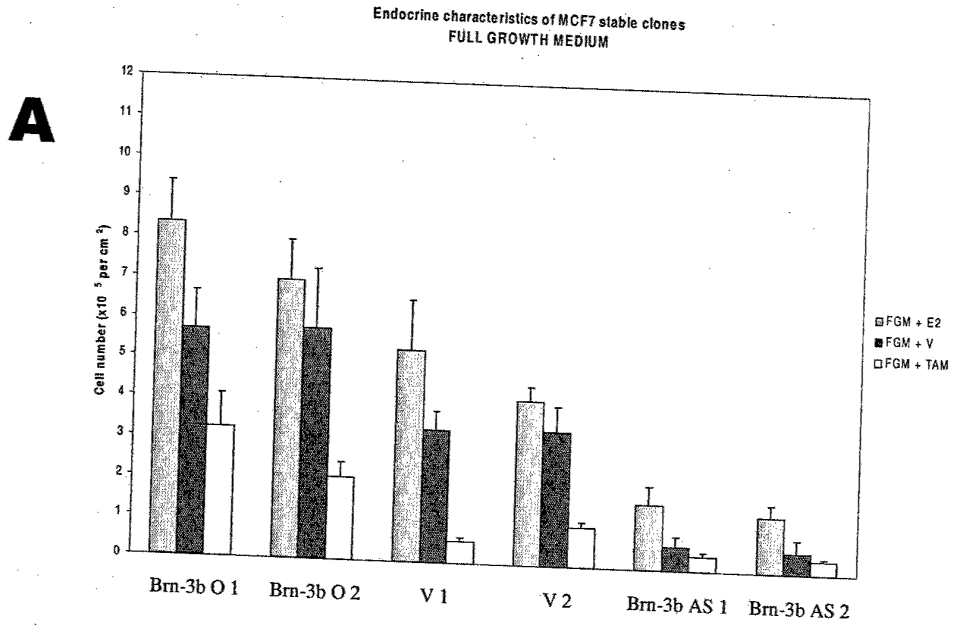

Tritiated thymidine incorporation of MCF7 stable clones
Counts per minute
4500
4000
3500
3000
2500
2000
1500
1000
500
0
*
*
*
*
Z
Y
B
C
A1
A2
MCF7 stable clone

Figure 6


Endocrine characteristics of MCF7 stable clones
FULL GROWTH MEDIUM
A
Cell number (x10 5 per cm 2)
0
1
2
3
4
5
6
7
8
9
10
11
12
Brn-3b O 1
Brn-3b O 2
V 1
V 2
Brn-3b AS 1
Brn-3b AS 2
FGM + E2
FGM + V
FGM + TAM

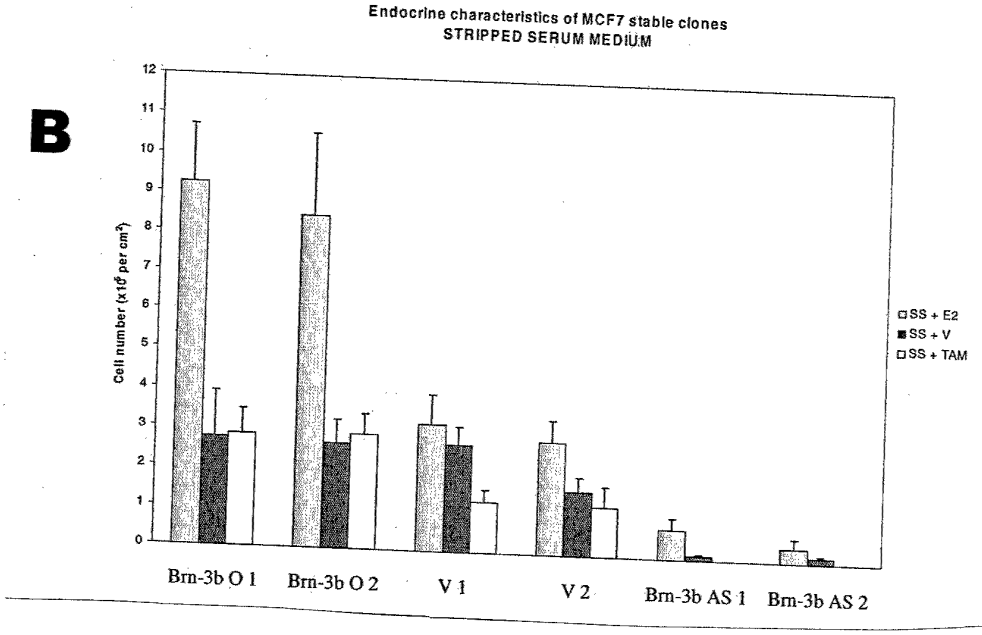

Endocrine characteristics of MCF7 stable clones
STRIPPED SERUM MEDIUM
B
Cell number (x10⁵ per cm²)
0
1
2
3
4
5
6
7
8
9
10
11
12
Brn-3b O 1
Brn-3b O 2
V 1
V 2
Brn-3b AS 1
Brn-3b AS 2
SS + E2
SS + V
SS + TAM

Figure 7

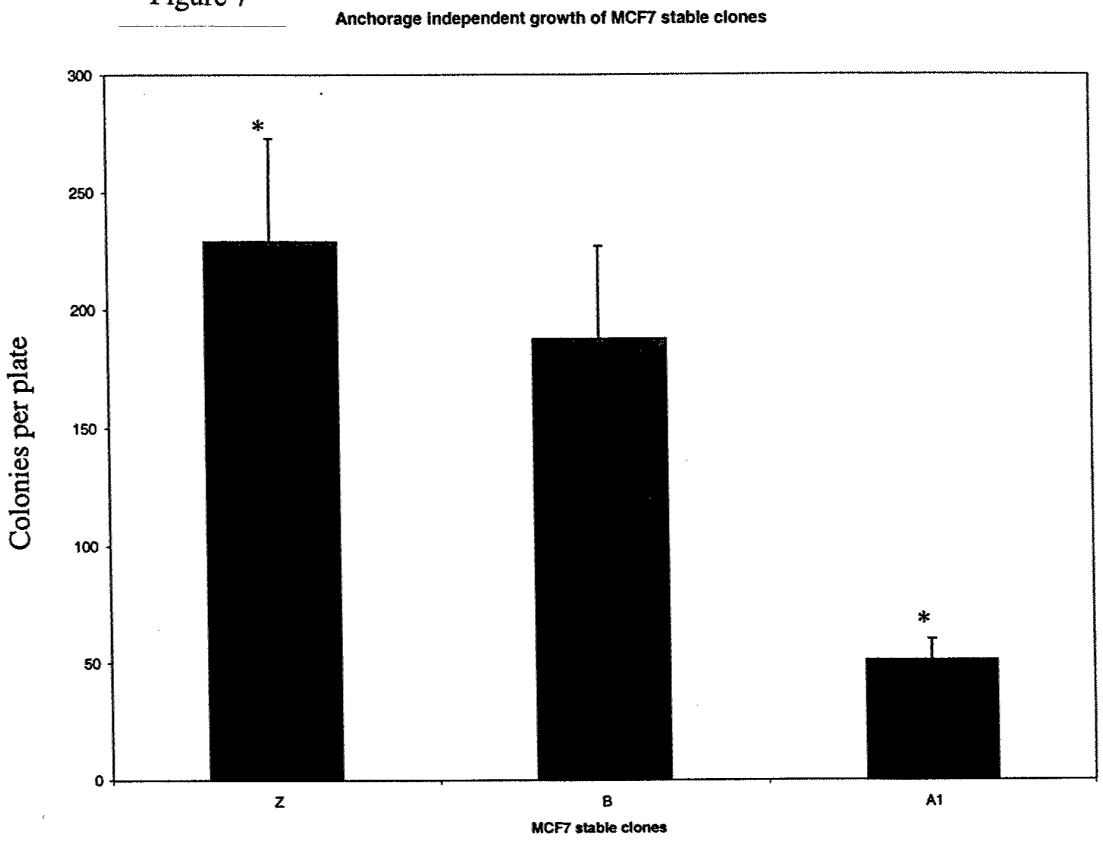
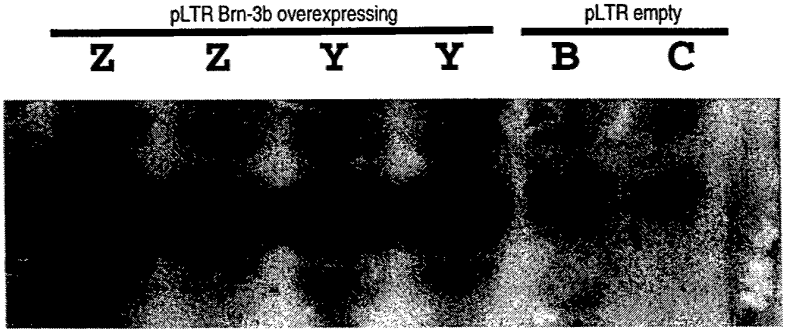


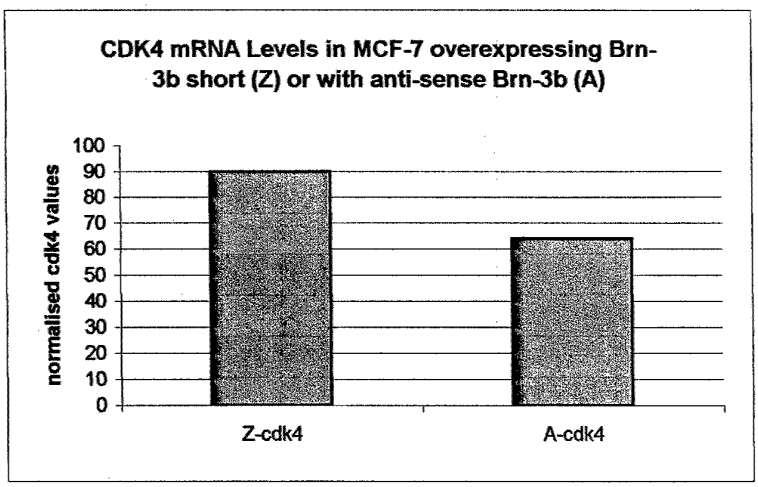
Figure 8

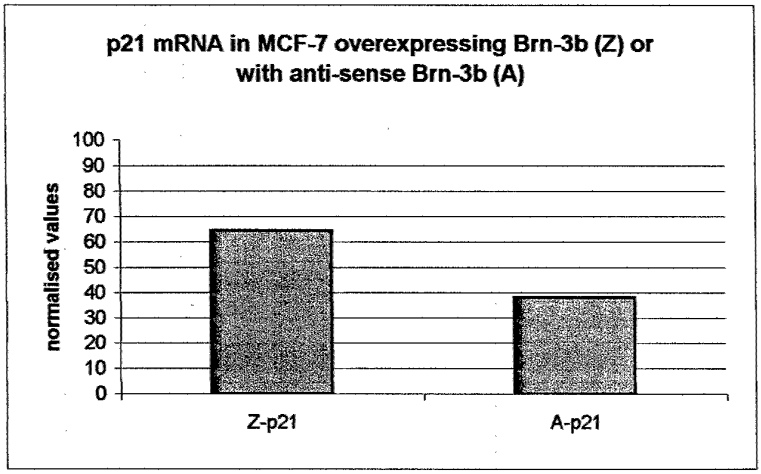


CLONE	DENSITOMETRY(arbitrary units)		FOLD EXPRESSION	
	beta hCG	Commassie	raw	normalized
pLTR Brn-3b short Z	1114033	551569	11.55	3.58
pLTR Brn-3b short Z	1047134	453077	10.86	4.10
pLTR Brn-3b short Y	1393001	350176	14.45	7.06
pLTR Brn-3b short Y	1255212	393675	13.02	5.66
pLTR B	130236	217177	1.35	1.06
pLTR C	62588	118621	0.65	0.94

Figure 9

Real-time RT-PCR results using LightCycler (values normalised with cyclophilin)





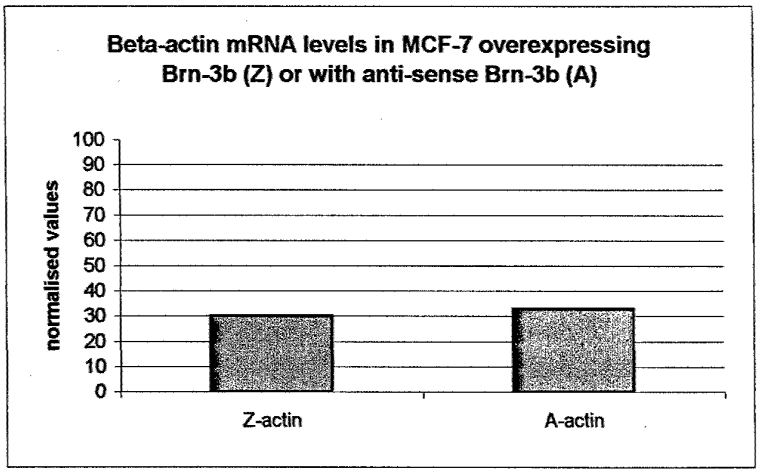
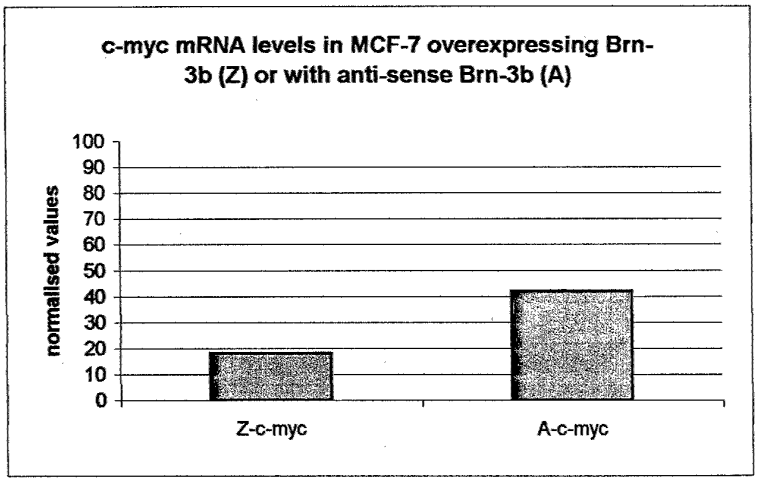
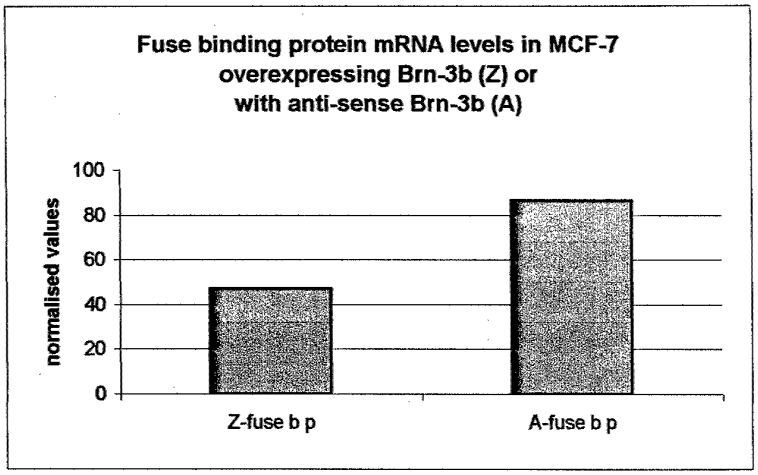


Figure 9

Real-time RT-PCR results using LightCycler (values normalised with cyclophilin)





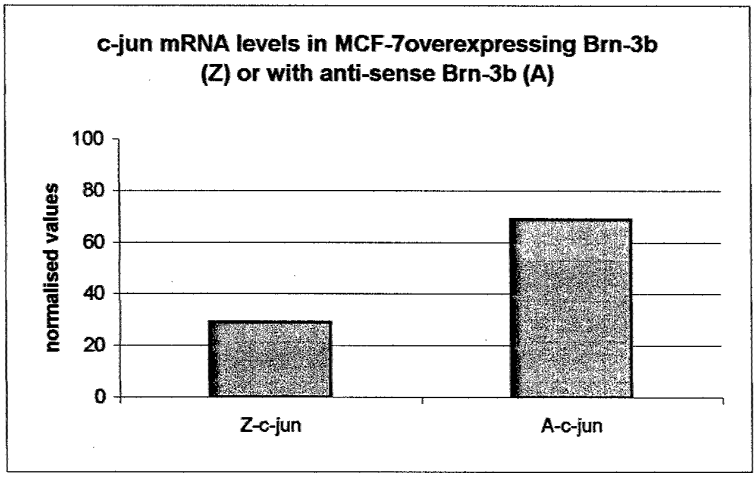
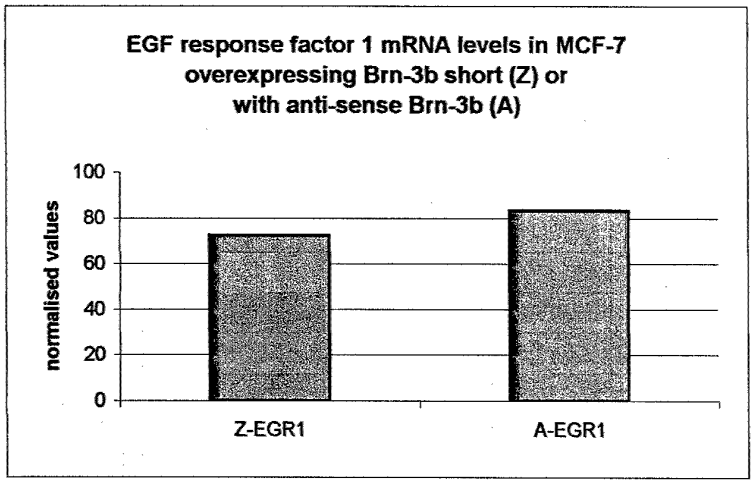
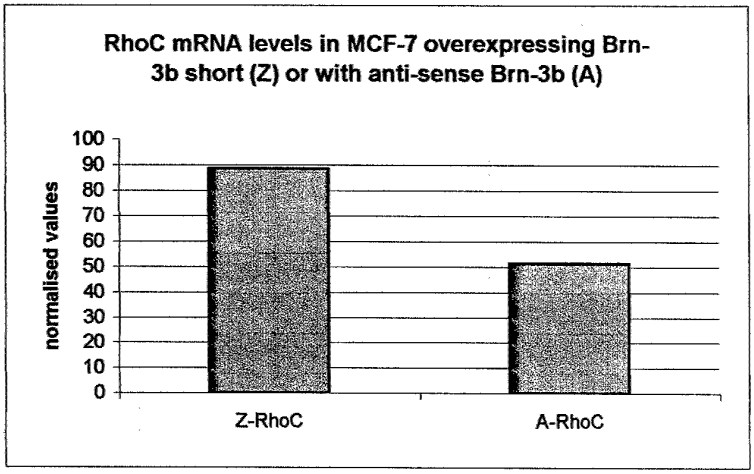


Figure 9

Real-time RT-PCR results using LightCycler (values normalised with cyclophilin)





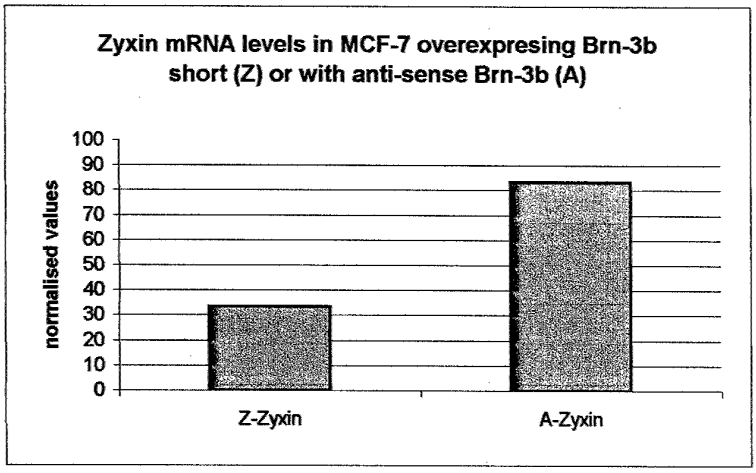


Figure 10

Real-time RT-PCR using MJ Opticon: Mean of all experiments