THE REGULATION OF ANNEXIN-A1'S EXPRESSION AND FUNCTION(S) BY 17-β-ESTRADIOL IN MCF7 BREAST CANCER CELLS

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ABBREVIATIONS USED

Anx-A1	Annexin-A1
Anx-A2	Annexin-A2
cDNA	Complementary DNA
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
E2	17-β-estradiol
EDTA	Ethylenediaminetetracetic acid
FBS	Fetal bovine serum
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
MAPK/ ERK	Mitogen-activated protein kinase/ extracellular signal-regulated kinase
MEK	MAPK/ ERK kinase
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide
PBS	Phosphate-buffered saline
RT-PCR	Reverse transcription-polymerase chain reaction
RNA	Ribonucleic acid
siRNA	Small interfering RNA
SDS-PAGE	Sodium dodecyl sulphate - polyacrylamide gel electrophoresis
TAE	Tris-acetate EDTA
TBS	Tris-buffered saline
TBST	Tris-buffered saline (with 0.05% Tween 20)

ABSTRACT

Estrogen, a naturally-occurring female steroid growth hormone, has been implicated as a major risk factor for the development of breast cancer. Recent research into this disease has also correlated Annexin-A1 (Anx-A1), a gluccocorticoid-inducible protein, with the development of breast tumorigenesis. Since both estrogen and Anx-A1 are implicated in breast cancer and have been shown in separate studies to be able to regulate the mitogen-activated protein kinase/ extracellular signal-regulated kinase (MAPK/ ERK) pathway, we hence hypothesize that estrogen (17-beta-estradiol/ E2) may induce the expression of Anx-A1 for the regulation of cell proliferation via the MAPK signalling pathway. Here, we show that prolonged exposure of MCF7 breast adenocarcinoma cells to high physiological levels of E2 led to an up-regulation of Anx-A1 expression and a corresponding inhibition of cell proliferation. In addition, Anx-A1 had direct anti-proliferative effects on cells, possibly via a constitutive over-activation of ERK. Finally, the anti-proliferative role of Anx-A1 was directly responsible, at least in part, for the reduced proliferation rates observed when cells were exposed to high E2 doses for an extended period of time. We thus believe that Anx-A1 may act as a tumor suppressor in cells and that its expression can be increased in times of need to put a brake on uncontrolled cellular proliferation.

(208 words)

1. INTRODUCTION

Breast cancer stands as one of the most common cancers among females and the second leading cause of cancer mortality for all women as of today[1]. As with all cancers, the cause of the disease is multi-factorial and several factors (including age, reproductive status and environmental insults) have been suggested to be associated with its development. In addition, there is also substantial evidence suggesting that high estrogen levels in postmenopausal women are associated with an increased risk to breast cancer, although the same association has not been established convincingly in pre-menopausal females (Bernstein and Ross, 1993). Yet, the mechanism by which estrogen may cause cancer remains unclear. It has been suggested that estrogen may promote breast cancer development by encouraging cell proliferation since it is a steroid growth hormone which promotes growth of breast tissues. In addition, studies have also shown that mammary tumors may be initiated by carcinogenic metabolites of estrogen which may react with DNA to induce oncogenic mutations (Devanesan *et al.*, 2001 and Fernandez e*t al.*, 2006). The link between estrogen and an increased risk of breast cancer is hence very real and significant.

With a high incidence of breast cancer mortality throughout the world population, it is not surprising that research into this area has been conducted extensively. In 1993, a research study conducted by Schwartz-Albiez *et al.* became one of the earliest studies to link breast cancer development to a particular group of proteins termed as annexins. In this study, a protein with a molecular weight of 38 kD was extracted from rats and human

[1] From the American Cancer Society – last revised on the 26th of September 2006

mammary tumor cell lines using affinity chromatography techniques and was subsequently identified as Annexin A2 (Anx-A2) using western blot analysis (Schwartz-Albiez *et al.*, 1993). Yet, interestingly, ensuing investigations showed that while Anx-A2 was found to be present in normal and tumor mammary gland cells, an evolutionarily related protein, Annexin A1 (Anx-A1), was found to be conspicuously absent in normal human mammary gland cells. *In vivo* studies on the expression of Anx-A1 in mammary tissues were thus conducted and it was discovered that within the acini and ductal myoepithelium, Anx-A1 was found only in normal and not tumorigenic mammary gland cells. Yet, in the stroma, Anx-A1 could only be found in tumor and not normal stromal cells (Schwartz-Albiez *et al.*, 1993). This differential expression of Anx-A1 in normal and malignant mammary cells thus highlights the possibility that Anx-A1 may be regulated differently in normal and tumorigenic tissues - a likelihood which was augmented when a study conducted by Ahn *et al.* in 1997 showed that Anx-A1 expression was not demonstrable in the ductal cells of normal tissues and benign tumors, but was generally present in malignant tissues (Ahn *et al.*, 1997).

The disparate expression of Anx-A1 in normal and malignant mammary cells may suggest that this protein could be regulated differently during the process of tumorigenesis, which could in turn imply that Anx-A1 may play a specific role in breast cancer development. While the exact physiological functions of Anx-A1 remain unclear, it has been suggested to play a part in many diverse functions, including cell proliferation, differentiation and apoptosis, all of which are intimately linked to the

process of cancer development. It is hence of interest for us to examine in greater detail, the possible linkage between Anx-A1 and breast cancer.

Anx-A1

Anx-A1 (also known as lipocortin-1) belongs to the annexin super family of proteins, which consists of a relatively large family of calcium and phospholipid-binding proteins that are evolutionarily conserved throughout animal and plant kingdoms (Gerke and Moss, 2002). By definition, an annexin protein has to fulfill two criteria – (1) it should be capable of binding (or '*annexing*') to negatively-charged phospholipids in a calcium-dependent manner, and (2) it has to contain a conserved structural element termed as the 'annexin repeat', a segment of approximately 70 amino acid residues (Gerke and Moss, 2002). In addition, each annexin protein is composed of two major domains – a divergent N-terminal region and the conserved C-terminal protein core. While the C-terminal core harbors the calcium and membrane binding sites, the N-terminal regions of annexins show much less similarity. It is not surprising hence, that the specific functions of different annexins are largely determined by their unique N-terminal regions (Nevid and Horseman, 1996).

Despite the fact that Anx-A1 makes up approximately 2 - 4% of total cytosolic protein in many cells and tissues, its endogenous physiological function(s) have remained largely unclear (Lim and Pervaiz, 2007). Nevertheless, many postulations have been proposed and unsurprisingly, most of them had focused on the anti-inflammatory and anti-migratory properties of Anx-A1 (Lim and Pervaiz, 2007), since it was first

discovered as a 37 kD protein factor which was induced by gluccocorticoids to suppress the inflammatory mediators of the ecosanoid family (Ahluwalia *et al*., 1996). Anx-A1 can thus, act as an endogenous anti-inflammatory protein which exerts its anti-inflammatory effects via down-regulation of phospholipase A_2 activity and the subsequent inhibition of phospholipase A_2-dependent inflammatory mediators such as prostaglandins and leukotrienes (Ahluwalia *et al*., 1996).

However, the anti-inflammatory effects of Anx-A1, albeit potent, are apparently not sufficient to represent the full spectrum of Anx-A1's potential physiological functions. In 1990, it was noted that proliferating fibroblasts induced Anx-A1 production by about three to four fold, while the expression levels of other annexins remained relatively low in dividing cells (Schlaepfer and Haigler, 1990). In 1996, the proliferative potential of Anx-A1 was further reiterated by studies which showed that phosphorylation of Anx-A1 could act as a signal transducer to amplify the proliferating signal of the hepatocyte growth factor (Skouteris and Schroder, 1996). Yet, the role of Anx-A1 in the regulation of cell proliferation is not without controversy. Various studies have also independently, demonstrated an inhibitory role for Anx-A1 in cell growth and proliferation. As early as 1993, Croxtall *et al.* had already shown that peptide fragments of Anx-A1 were able to inhibit growth of lung cancer cells and block epidermal growth factor-induced stimulation of cell proliferation (Croxtall *et al*., 1993). A decade later, Alldridge and Bryant also reported that Anx-A1 could inhibit cell growth by disrupting the actin cytoskeleton and inhibiting cyclin D1 expression via a constitutively sustained activation of the ERK 1/2 MAPK signal (Alldridge and Bryant, 2003).

Various other possible roles of Anx-A1 have also been suggested. In 2000, Rhee *et al.* reported that Anx-A1 could perhaps act as a stress protein as its expression was strongly induced upon cellular stresses such as heat and chemicals (Rhee *et al.*, 2000). In 2003, yet another plausible function of Anx-A1 was discovered – that of cellular apoptosis. Arur *et al.* had observed that Anx-A1 acted as an endogenous ligand which mediated apoptotic cell engulfment in a caspase-dependent manner both *in vitro* in various cell lines, and *in vivo*, in the nematode *C. elegans* (Arur *et al.,* 2003).

It should be noted however, that although various potential functions of Anx-A1 have been suggested in different studies, the exact physiological function(s) of Anx-A1 remain to be elucidated. Work in this direction is hence important, especially if we are to achieve a deeper understanding of the possible role(s) of Anx-A1 in tumorigenesis.

Anx-A1 and cancer

Since its discovery as a major cellular substrate for tyrosine phosphorylation by the epithelial growth factor (EGF) receptor, Anx-A1 has been implicated in various pathways known to be subverted or involved in cancer (Gerke and Moss, 2002). One of this is the ERK cascade - a major cell signalling pathway involved in the regulation of cell proliferation (Alldridge and Bryant, 2003). However, research in this area has been conflicting, and the major difficulty comes in the reconciliation of all the diverse reported effects of Anx-A1 on MAPK signalling, calcium-ions mobilization and apoptosis *etc*. (Gerke and Moss, 2002). While Anx-A1 has been shown to be strongly down-regulated in prostate cancer (Xin *et al.*, 2003), head and neck cancer (Garcia Pedrero *et al*., 2004)

and oesophageal cancer (Hu *et al.*, 2004), it was observed to be up-regulated in hepatocarcinoma (De Coupade *et al.*, 2000) and pancreatic cancer (Bai *et al.*, 2004). Clarification of these seemingly contradictory data will thus require much future work.

An important point to note is that while the correlation between cancer development and the up/down-regulation of Anx-A1 may appear striking, evidence in support of any causative role(s) for Anx-A1 in the development of the disease is still mainly circumstantial (Gerke and Moss, 2002) and requires further investigation. It is thus, our objective to examine the possible role(s) of Anx-A1 in breast cancer. In this study, we will hence specifically look at the possible regulation of Anx-A1's expression and function(s) by estrogen in the breast cancer cell line MCF7.

The possible linkage between estrogen, Anx-A1 and breast cancer development

It was mentioned earlier that prolonged exposure to estrogen has been identified as a major risk factor in the development of breast cancer and that Anx-A1 has also been implicated in the development of breast tumorigenesis. However, reports on the exact role of Anx-A1 in breast cancer have been conflicting. While earlier studies have shown Anx-A1 to be up-regulated in mammary adenocarcinoma (Ahn *et al.*, 1997, Pencil and Toth, 1998), a recent report has demonstrated that a decreased Anx-A1 expression was correlated with development and progression of the disease, as determined by a tissue microarray analysis (Shen *et al.*, 2006). Although it has been suggested that the contradictory reports on the expression of Anx-A1 in breast cancers may be correlated with estrogen receptor status (Lim and Pervaiz, 2007), an obvious association has not

been established. It is hence of interest for us to determine if Anx-A1 expression can be regulated by estrogen, since if Anx-A1 expression can indeed be induced by estrogen, disparate levels of estrogen and differing estrogen receptor status among individuals may well account for the inconsistencies observed with regards to Anx-A1 levels and breast cancer development.

In addition to the fact that both estrogen and Anx-A1 have been linked to the development of breast tumorigenesis, another reason which suggests a possible linkage between the two is that Anx-A1 was first discovered as a gluccocorticoid-inducible factor. It is hence plausible that estrogen, being a naturally-occurring female steroid growth hormone chemically related to gluccocorticoids, may be able to induce the expression of Anx-A1 as well.

Moreover, both Anx-A1 and estrogen have been implicated in various independent studies, in the regulation of the MAPK/ ERK signalling pathway – a pathway often dysregulated in the events leading to the development of the cancer phenotype. The core of the MAPK cascade consists of an evolutionarily conserved module of three sequentially activated protein kinases, whereby catalytic activation of the MAP kinase (MAPK) requires phosphorylation by an upstream MAPK kinase (MAP2K/ MAPKK/ MEK), which is in turn, activated by yet another kinase upstream of itself (MAP2K kinase/ MAP3K/ MAPKKK/ MEKK) (Wilkinson and Millar, 2000). Since the activation of effector MAP kinases (the best characterized of which, belong to the mammalian extracellular signal-regulated kinase (ERK) family) results in multiple effects

ranging from the phosphorylation of cytoplasmic targets to the modulation of transcription factors that regulate gene expression (Wilkinson and Millar, 2000), it is not surprising that the activation/ inactivation kinetics of the MAPK signalling pathway have been associated with various biological responses, including cell proliferation and transformation (Alldridge and Bryant, 2003). As the functions of both Anx-A1 and estrogen may converge via this common pathway, it is hence possible that estrogen may somehow regulate the expression of Anx-A1 during its course of action in the cell.

We thus hypothesize that 17-beta-estradiol (E2), the major estrogen synthesized by the ovaries of the human female, can induce Anx-A1 expression in estrogen receptor-positive breast adenocarcinoma cells (MCF7 cells), and that this induction may have a role in regulating cell proliferation. Research into this area can hence enhance our understanding of the possible role(s) of Anx-A1 in tumorigenesis, which is at present, fairly limited as evidence in support of any causative role(s) for Anx-A1 in the development of the disease is still mainly circumstantial (Gerke and Moss, 2002). At the same time, such investigations can also perhaps, pave the way for novel therapeutics for the disease via targeting of the protein and/or lead to the possible identification of Anx-A1 as a potential biomarker in breast cancer development and progression.

2. OBJECTIVES OF STUDY

(1) To investigate if E2 can regulate Anx-A1 expression levels in MCF7 breast cancer cells and if so,

(2) To investigate if the regulation of Anx-A1 by E2 has a physiological function

(3) To examine the possible role(s) of Anx-A1 in breast tumorigenesis

3. MATERIALS AND METHODS

3.1 Tissue Specimens

Breast tissue surgical samples were obtained from the Tissue Repository Centre of the National University Hospital (NUH). Tumour and matched control samples were requested from female patients who had not undergone any drug treatment. Samples collected were immediately homogenized and proteins were extracted on the day of collection.

3.2 Tissue Homogenization and Protein Extraction

50-100 mg of tissue sample was homogenized in 1 ml of TRIzol Reagent (Invitrogen, Life Technologies) using a polytron power homogenizer. TRIzol Reagent is a ready-to-use reagent for the isolation of total RNA, DNA and proteins from cells and tissues. Insoluble material was then removed from the homogenate by centrifugation at 12,000 x g at 2 to 8^{o}C. Excess fats at the top layer were removed and the cleared homogenate solution was transferred to a fresh tube.

Homogenate solutions were incubated for 5 min at room temperature and 0.2 ml of chloroform (Sigma Aldrich, St. Louis, USA) per 1 ml of TRIzol Reagent was then added. The tubes were shaken vigorously by hand for 15 s and incubated at room temperature for another 2 to 3 min. The tubes were next centrifuged at 12,000 x g for 15 min at 2 to 8^{o}C. Following centrifugation, the mixture separated into a lower red, phenol-chloroform phase, an interphase and a colourless upper aqueous phase.

The upper aqueous phase (containing RNA) was removed completely and 0.3 ml of absolute ethanol was next added. The samples were then incubated at room

temperature for 2 to 3 min and the DNA from the tissues was sedimented by centrifugation at 2,000 x g for 5 min at 2 to 8^{o}C.

Proteins were removed from the phenol-ethanol supernate with 1.5 ml of isopropyl alcohol. The samples were kept at room temperature for 10 min and the protein precipitate was sedimented at 12,000 x g for 10 min at 2 to 8^{o}C. The supernatant was removed and the protein pellet was washed three times in 2 ml of a solution containing 0.3 M guanidine hydrochloride in 95% ethanol. During each wash cycle, the protein pellet was stored in the wash solution for 20 min at room temperature and then centrifuged at 7,500 x g for 5 min at 2 to 8^{o}C. After the final wash, the protein pellet was vortexed in 2 ml of absolute ethanol. The protein pellet was stored in ethanol for 20 min at room temperature and centrifuged at 7,500 x g for 5 min at 2 to 8^{o}C. The protein pellet was next vacuum-dried for 5 to 10 min and dissolved in 1% SDS. Samples were later analyzed by western immunoblotting or stored at -20^{o}C for future use.

3.3 Cell Culture

The human breast adenocarcinoma cell lines (MCF7 and MDA-MB-231) were obtained from the American Type Culture Collection (ATCC) and cultured in RPMI 1640 media (Hyclone Laboratories) supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine and 1% penicillin-streptomycin (GIBCO-BRI, Gaithersburg MD, USA). These monolayer cells were maintained in a 37^{o}C incubator with 5% CO_2. Cells were checked regularly under the light microscope and were sub-cultured when they reached 70-80% confluence.

24 hrs prior to drug treatment, cells were incubated with charcoal-treated cell culture medium. The constituents of the charcoal-treated medium were exactly the same as those mentioned above except that the 10% FBS was replaced by 10% charcoal-treated FBS. Charcoal-treated FBS was made by adding 0.5 g of activated charcoal (Sigma Aldrich, St. Louis, USA) to 50 ml of sterile FBS. The FBS was then left to stand at room temperature for one hour before it was centrifuged at 3,000 rpm for 10 min. The supernatant was removed and the above steps were repeated for a total of three times before the FBS was syringe-filtered and stored at -20^{o}C under sterile conditions.

MCF7 cells are estrogen-receptor positive breast cancer cells which have been shown previously to express Anx-A1. This thus supports the use of MCF7 cells in this study. In contrast, MDA-MB-231 cells lack the estrogen receptor-alpha, and were hence, used as a control in our experiments.

3.4 Drug Treatment

Two days prior to drug treatment, 10^6, 800,000 or 650,000 cells were seeded in 25 cm^2 flasks in normal cell culture medium (for drug treatment over 24, 48 or 72 hrs respectively). One day later, the medium was replaced by charcoal-treated cell culture medium. The treatment of cells with charcoal-treated medium is a well-established method commonly employed in studies involving the usage of steroid hormones. Since the activated charcoal would absorb the hormones present in the FBS, the pre-treatment of cells in charcoal-treated medium for a short while before drug treatment would allow for the removal of confounding factors, namely, the presence of other endogenous steroid hormones (such as estrogen and gluccocorticoids) within the FBS of the cell culture

medium. This is especially important in our study as it was observed by previous groups that the presence of endogenous gluccocorticoids in serum induced large amounts of Anx-A1 which in turn, masked any subsequent gluccocorticoid-induction of Anx-A1 (Croxtall and Flower, 1992).

On the day of drug treatment, cells were incubated in fresh charcoal-treated medium. 17-beta-estradiol (Sigma Aldrich, St. Louis, USA) and/or tamoxifen (Sigma Aldrich, St. Louis, USA) were added at final concentrations of 10^{-7} M, 10^{-9} M and 10^{-11} M. These concentrations were chosen because they represent the general physiological concentrations of estrogen in reproductive and post-reproductive females. 17-beta-estradiol (E2) is one of the three naturally-occurring estrogens in the human body. It was utilized in our study because it is the most potent estrogen of the three and also, because it is the major estrogen synthesized by the ovaries of the human female. Tamoxifen was used because it is known to have both mildly estrogenic and anti-estrogenic effects on cells.

Cells were harvested at different time-points (24 hrs, 48 hrs and 72 hrs) after drug treatment.

3.5 MAPK Inhibition Studies

For MAPK inhibition studies, cells were pre-treated with a MEK inhibitor, U0126 (Promega), in solution or with DMSO as a control. On the day of drug treatment, MCF7 cells were first serum-starved for 2 hrs before being incubated with 10 mM of U0126 for 1.5 hrs. 10% charcoal-treated FBS and appropriate drug concentrations were then added before cells were maintained under normal cell culture conditions mentioned previously.

3.6 Extraction of Total Cellular Protein

Following drug treatment, cells were washed with ice cold 1X PBS and immediately placed at -20^{o}C for 5 min. This procedure is essential in maintaining the phosphorylation status of proteins of interest. The cells were then scraped in the presence of a lysis buffer (200 µl per 3 x 10^6 cells) which consists of 10 mM HEPES (pH 7.9) (Sigma Aldrich, St. Louis, USA), 10 mM KCl (Merck & Co., Inc.), 0.1 mM EDTA, 10% NP40, 1X protease inhibitor (Pierce Biotechnology), phosphatase inhibitor – 50 µM okadaic acid (Sigma Aldrich, St. Louis, USA) and 200 mM sodium vanadate (Sigma Aldrich, St. Louis, USA). This mixture was kept on ice at all times and vortexed for 1 min every half an hour. After 2 hrs, the lysate was centrifuged at 12,000 x g for 20 min at 4^{o}C. The supernatant was then collected and subsequently stored at -20^{o}C until evaluation by SDS-PAGE analysis.

3.7 Fractionation of Cells

In some experiments, cells harvested following drug treatment were also fractionated into membranal, cytoplasmic and nuclear fractions to allow for the study of Anx-A1 in different cellular fractions. The cytoplasmic fractions were then analyzed for Anx-A1 expression.

Cells were washed twice with ice cold 1X PBS and resuspended in 400 µl of a buffer consisting of 10 mM Hepes (pH 7.9) (Sigma Aldrich, St. Louis, USA), 10 mM KCl, 1.5 mM $MgCl_2$ and 1X protease inhibitor (Pierce Biotechnology) for 15 min. 25 µl of NP40 lysis buffer (Sigma Aldrich, St. Louis, USA) was then added and the mixture was vortexed for 15 seconds before being left to stand on ice for 1 min. This mixture was

centrifuged at 16,000 x g for 5 min at 4°C. The supernatant, consisting of the cytoplasmic and membranal fractions, was further spun using ultra-centrifugation at 100,000 x g for 1 hr. After spinning, the supernatant containing the cytoplasm was collected and transferred to a fresh tube. All cytoplasmic samples were stored at -20°C until further evaluation.

3.8 Protein Determination

5 µl of protein albumin standards with known protein concentrations (Pierce Biotechnology) or 1 µl of protein sample was added to 150 µl of Comassie Plus™ Protein Assay Reagent (Pierce Biotechnology). Absorbance was measured at a wavelength of 595 nm and the absorbance intensities of the protein standards were plotted to obtain a standard curve. Absorbance intensities of protein samples were then read off the standard curve to determine the amount of protein within the sample.

3.9 SDS-PAGE and Western Immunoblot Analysis

Equal amounts of protein from each sample were subjected to 15% SDS-PAGE at a constant voltage of 125 V. The proteins were then transferred onto nitrocellulose membranes (Bio-Rad Laboratories) using a wet transfer apparatus (Bio-Rad Laboratories).

Following transfer, the membranes were washed with distilled water to remove traces of transfer buffer and then air-dried for several hours to allow for firm binding of proteins to membranes. The membranes were rewetted with 1X TBS and blocked with 5% w/v milk proteins in 1X TBST (BLOTTO) for 1 hr. Membranes were then washed for 15 min with 1X TBST (TBS with 0.05% Tween 20) before incubation with the

appropriate primary antibody (see table below) at 4^{o}C overnight. Membranes were next washed thrice with 1X TBST for a total of 30 min before incubation with secondary antibodies (see table below) conjugated to horseradish peroxidase for 1 hr. Three washes lasting 10 minutes each were carried out prior to the addition of the chemiluminiscent West Pico Substrate (Pierce Biotechnology). Blots were then exposed to X-ray films until bands were clearly seen after film development.

Primary Antibody	**Dilution**	**Secondary Antibody (HRP conjugated)**	**Dilution**
Monoclonal mouse anti-Anx-A1 antibody (Biosource)	1:3000	Goat anti-mouse IgG (Pierce Biotechnology)	1:5000
Monoclonal mouse anti-p21$^{waf/cip}$ antibody (Santa Cruz)	1:1000	Goat anti-mouse IgG (Pierce Biotechnology)	1:5000
Polyclonal rabbit anti-phosphorylated p44/42 MAPK antibody (Cell Signaling Technology)	1:2000	Goat anti-rabbit IgG (Pierce Biotechnology)	1:5000
Polyclonal rabbit anti-p44/42 MAPK antibody (Cell Signaling Technology)	1:2000	Goat anti-rabbit IgG (Pierce Biotechnology)	1:5000
Monoclonal mouse anti-alpha-tubulin antibody (Santa Cruz)	1:5000	Goat anti-mouse IgG (Pierce Biotechnology)	1:5000
Monoclonal mouse anti-beta-actin antibody (Santa Cruz)	1:5000	Goat anti-mouse IgG (Pierce Biotechnology)	1:5000

3.10 Western Blot Quantification

Where applicable, western blots were scanned at 600 dpi for densitometric quantification, using the NIH Image 1.37 software. Western blot densitometric values were then normalized with that of a control band (alpha-tubulin or ERK 1) so as to compare changes in protein expression or activation levels. Although the quantification of proteins by western blot densitometry reflects the nature of an indirect measurement method and does not directly indicate actual amounts of detected proteins, these measurements do however provide informative comparisons for relative amounts of proteins of interest.

3.11 RNA Extraction

Cells grown in each 25 cm^2 flask were lysed directly by adding 2.5 ml of TRIzol Reagent (Invitrogen, Life Technologies), and passing the cell lysate through a pipette. Samples were then incubated for 5 min at room temperature and 0.2 ml of chloroform (Sigma Aldrich, St. Louis, USA) per 1 ml of TRIzol Reagent was added. The tubes were shaken vigorously by hand for 15 s and incubated at room temperature for another 2 to 3 min. The tubes were next centrifuged at 12,000 x g for 15 min at 2 to 8^oC. Following centrifugation, the mixture separated into a lower red, phenol-chloroform phase, an interphase and a colourless upper aqueous phase. The RNA which remained exclusively in the aqueous phase was then transferred into a fresh tube.

RNA was precipitated from the aqueous phase by adding 0.5 ml of isopropyl alcohol per 1 ml of TRIzol reagent used for the initial cell lysis. Samples were then incubated at room temperature for 10 min and centrifuged at 12,000 x g for 10 min at 2 to

8°C. Following removal of the supernatant, the RNA pellet was washed once with 1 ml of 75% ethanol per 1 ml of TRIzol Reagent used for the initial cell lysis. Samples were then mixed by vortexing and centrifuged at 7,500 x g for 5 min at 2 to 8°C. At the end of the procedure, the RNA pellet was air-dried for 5 to 10 min and dissolved in RNase-free water. All RNA samples were stored at -70°C and analyzed within a week by subsequent RT-PCR and DNA gel electrophoresis analysis.

3.12 RT-PCR and DNA Gel Electrophoresis

RT-PCR was performed on total RNA extracted from MCF7 cells using the Qiagen® OneStep RT-PCR Kit (Qiagen Inc., CA, USA), which contains optimized components that allow both reverse transcription and polymerase chain reaction amplification to take place in a "one-step" reaction. Procedures were carried out in accordance to manufacturer's instructions, using 1 μg of template RNA per reaction. (RNA concentration was determined by using a spectrophotometer with an ultraviolet absorbance set at 260 nm). The following primers were used:

Anx-A1 Forward Primer (1st Base):
5'-CTG GAA GCT TTG GTA TCA GAA TTC CTC AAG C-3'

Anx-A1 Reverse Primer (1st Base):
5'-TCC TCC TAG AGT TTC CTC CAC AAA GAG CC-3'

GAPDH Forward Primer (1st Base):
5'-AAC ACA GTC CAT GCC ATC AC-3'

GAPDH Reverse Primer (1st Base):
5'-TCC ACC ACC CTG TTG CTG TA-3'

PCR was performed using the following parameters: 1 cycle for 30 min at 50^{o}C for reverse transcription; 1 cycle for 15 min at 95^{o}C for initial PCR activation; 25 or 29 cycles at 94^{o}C, 68^{o}C and 72^{o}C for 1 min each (for GAPDH and Anx-A1 cDNA amplification respectively); 1 cycle for 10 min at 72^{o}C for the final extension; and 1 cycle at 4^{o}C for overnight storage.

PCR products were separated by 1.5% agarose gel electrophoresis in 1X TAE electrophoresis buffer and visualized by ethidium-bromide staining. The resulting florescent bands were then video-digitalized by a GelDoc 1000UV-Gel camera and PCR products were identified as a single band corresponding to expected molecular sizes.

3.13 Transfection Studies

For functional studies involving Anx-A1, MCF7 cells were transfected with either Anx-A1 siRNA or a plasmid expressing Anx-A1, to produce cells with lower or higher Anx-A1 levels (as compared to normal cells) respectively.

3.13.A Transfection with Anx-A1 siRNA

Cells were transfected with Anx-A1 siRNA (Dharmacon *SMART*pool® siRNA) to produce MCF7 cells with lower levels of Anx-A1 as compared to normal MCF7 cells. Two sets of control experiments were also performed, whereby the cells were either transfected with scrambled ANX-A1 siRNA (Qiagen Inc., CA, USA), or the transfection reagent – oligofectamine (Invitrogen, Life Technologies) alone. (Scrambled siRNA sequence: sense strand – r(GGG GAC AUA CGU AAA CGU G)dTdT; anti-sense strand: r(CAC GUU UAC GUA UGU CCC C)dTdT)

On the day before transfection, 120,000 MCF7 cells were seeded in each well of a 6-well plate. 3 hrs prior to transfection, the cell culture medium was replaced with serum-free RPMI. 5 μl (100 nM) of siRNA and 12 μl of oligofectamine were then separately added to 70 μl of serum-free RPMI in the absence of antibiotics. This was then mixed gently and incubated at room temperature for 20 min to allow for the formation of the siRNA-oligofectamine complex. The cells were next incubated with the siRNA-oligofectamine complexes in a 37^{o}C incubator supplemented with 5% CO_2 for 5 hrs, after which, 2 ml of normal cell culture medium was added. Transfected cells were then maintained in the incubator overnight, before being subjected to further tests or for protein extraction.

3.13.B Transfection with pcDNA3.1-V5 plasmid

To produce MCF7 cells that over-express Anx-A1, a pcDNA3.1-V5 plasmid[2] expressing human Anx-A1 was used. The Anx-A1 was amplified by PCR using human peripheral blood mononuclear cells' cDNA and PCR primers, and was then cloned in frame (without the stop codon) with the V5 tag at the COOH terminus.

MCF7 cells were transfected using the same procedure as described above, except that the siRNA was replaced with the plasmid vector and that the transfection reagent used was replaced with SuperFect transfection reagent (Qiagen Inc., CA, USA). Cells transfected with an empty pcDNA3.1 plasmid and cells transfected with the SuperFect transfection reagent alone were used as controls.

[2] The plasmid was kindly provided by Professor Fulvio D'Acquisto from the William Harvey Research Institute, Queen Mary's School of Medicine and Dentistry, London.

3.14 Cell Proliferation Assays

Both Cell Titer 96® Aq_{ueous} One Solution Cell Proliferation Assay (Promega) and alamarBlue® assay (Biosource) are colorimetric methods used for the determination of the number of viable cells in proliferation assays. While the former contains a novel tetrazolium compound which is bio-reduced by cells to a colored formazan product that is soluble in cell culture medium, the alamarBlue® assay incorporates an oxidation-reduction indicator that both fluoresces and changes color in response to chemical reduction of growth medium resulting from cell growth.

3.14.A Cell Titer 96® Aq_{ueous} One Solution Cell Proliferation Assay

1,500 cells were plated in each well of a 96-well plate and left to adhere for a minimum of 4 hrs, after which the cell culture medium was aspirated and replaced with 100 µl of charcoal-treated cell culture medium. After 24 hrs, this was replaced with fresh charcoal-treated medium and drugs were added in the appropriate concentrations. After drug treatment, 20 µl of Cell Titer 96® Aq_{ueous} One Solution reagent was added into each well and the plates were left to incubate in a 37°C incubator with 5% CO_2 for 3 hrs. The absorbance intensities were then measured at 490 nm and the cell proliferation relative to the control sample was calculated using the formula below:

$$\frac{\text{Mean of triplicate optical density values of test sample}}{\text{Mean of triplicate optical density values of control}} \times 100\%$$

3.14.B alamarBlue® Assay

1,500 cells were plated in each well of a 96-well plate and left to adhere for a minimum of 4 hrs, after which the cell culture medium was aspirated and replaced with

200 µl of charcoal-treated cell culture medium. After 24 hrs, this was replaced with fresh charcoal-treated medium and drugs were added in the appropriate concentrations. 1 day following the addition of drugs, 20 µl of alamarBlue® reagent was added into each well and the plates were left to incubate in a 37°C incubator with 5% CO_2. The proliferation status of cells was continuously monitored over a period of up to 5 days, with absorbance intensities being measured at two different wavelengths of 570 nm and 600 nm everyday. Cell proliferation relative to the control sample was calculated using the formula below:

$$\frac{(117,216 \times A_{570} \text{ of test sample}) - (80,586 \times A_{600} \text{ of test sample})}{(117,216 \times A_{570} \text{ of control}) - (80,586 \times A_{600} \text{ of control})} \times 100\%$$

where 117, 216: Molar Extinction Coefficient of oxidized alamarBlue® dye measured at a wavelength of 600 nm (value given by manufacturer);

80, 586: Molar Extinction Coefficient of oxidized alamarBlue® dye measured at a wavelength of 570 nm (value given by manufacturer);

A_{570}: absorbance measured at 570 nm and

A_{600}: absorbance measured at 600 nm.

All samples for cell proliferation assays were analyzed in triplicates.

3.15 MTT Cell Viability Assay

Cell viability under experimental conditions was determined by an assay that employed mitochondria-dependent reduction of 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide (MTT) to formazan. 0.2-0.3 million cells were plated onto

each well of a 24-well plate prior to drug treatment. Following drug treatment, cells were dislodged via vigorous pipetting. 50 µl of the cells in their cell culture medium were then placed in each well of a 96-well plate. Cells were next pulsed with 50 µl of freshly prepared MTT solution (3-5 mg of MTT reagent (Sigma Aldrich, St. Louis, USA) dissolved in plain RPMI) per sample in triplicates for 2 hrs at 37^{o}C in the dark. After incubation, the plate was spun at 3,000 rpm for 5 min and the supernatant was removed. The crystals were then dissolved in 200 µl of DMSO and 10 µl of Sorenson's glycine buffer. The absorbance intensities were measured at 570 nm and the cell viability relative to the control sample was calculated using the formula below:

$$\frac{\text{Mean of triplicate optical density values of test sample}}{\text{Mean of triplicate optical density values of control}} \times 100\%$$

3.16 Annexin V-FITC Apoptosis Detection

The Annexin V-FITC Apoptosis Detection Kit I (BD Biosciences Pharmingen) makes use of the ability of cells to lose membrane asymmetry in the early phases of apoptosis to quantitatively determine the percentage of cells that are actively undergoing apoptosis.

Following drug treatment, the cells were harvested via incubation with 1 mM of EDTA dissolved in 1X PBS. The cells were then washed twice with ice cold 1X PBS and resuspended in 1X binding buffer at a concentration of 10^6 cells/ ml. 100 µl of the cells (10^5 cells) were then transferred to a 5 ml culture tube. 5 µl of Annexin V-FITC and 5 µl of propidium iodide (PI) were added. The cells were then gently vortexed and incubated

in the dark at room temperature. 400 µl of 1X binding buffer was added to each tube and the samples were analyzed by flow cytometry within 1 hr.

3.17 Statistical Data Analysis

In cases where multiple duplications were possible, data are represented as means ± SEM (standard error of the mean). Individual groups were compared using the Student's *t* test with a two-tailed *p* value. A value of $P < 0.05$ was taken as significant.

4. RESULTS

4.1 Anx-A1 is differentially expressed in tumor and normal tissues taken from breast surgical samples

Because few studies have been done on Anx-A1 expression in breast cancer, we first seek to confirm that Anx-A1 is indeed differentially expressed in normal and tumor samples in the first place. As a confirmatory study, breast tumor tissue samples (with matched controls) were collected from four patients and the extracted proteins were analyzed by western immunoblotting. Out of the four, three patients had Anx-A1 expressed at different levels between normal and tumorigenic tissues, although there was no sign that Anx-A1 was consistently up or down-regulated in all three samples (Figure 1).

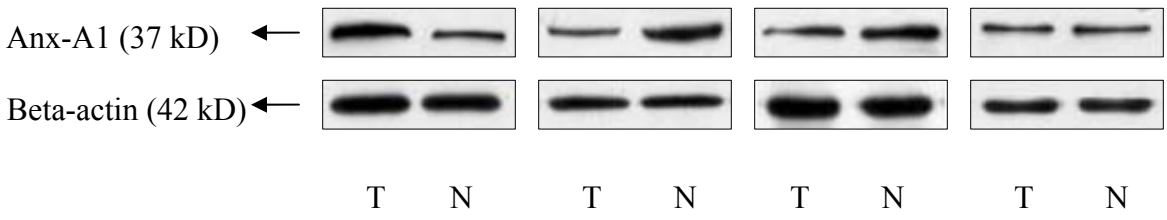


Figure 1: Western blot analysis of Anx-A1 expression in tumor and matched control samples. Anx-A1 was differentially expressed in three out of the four breast surgical samples collected (the first three samples from the left). Beta-actin was used as a control to ensure equal loading of protein samples during analysis. T: tumorigenic tissue sample; N: normal tissue (matched control) from the same patient.

4.2 E2 increases cytosolic Anx-A1 levels in MCF7 cells in a dose-dependent manner after 72 hrs

There are three pools of Anx-A1 which are known to be present in cells, namely cytosolic, nuclear and membrane-associated Anx-A1. However, as the levels of nuclear and membranal Anx-A1 in MCF7 cells are so low as to be virtually undetectable (data not shown), this study will focus on the cytosolic and total Anx-A1 present in MCF7 cells.

The exogenous addition of E2 had no apparent effects on cytosolic Anx-A1 in MCF7 cells after 24 or 48 hrs, as shown by western blot analysis (Figures 2 and 3). However at 72 hrs, changes in Anx-A1 levels were observed, with a higher level of Anx-A1 corresponding with a higher dose of E2 used (Figure 4). Interestingly, while cells treated with tamoxifen showed no increase in Anx-A1, cells treated with both E2 *and* tamoxifen showed an increase in cytosolic Anx-A1. It thus appears that 10^{-9} M of tamoxifen was unable to inhibit the expression of Anx-A1 induced by the same concentration of E2 used.

24 hrs

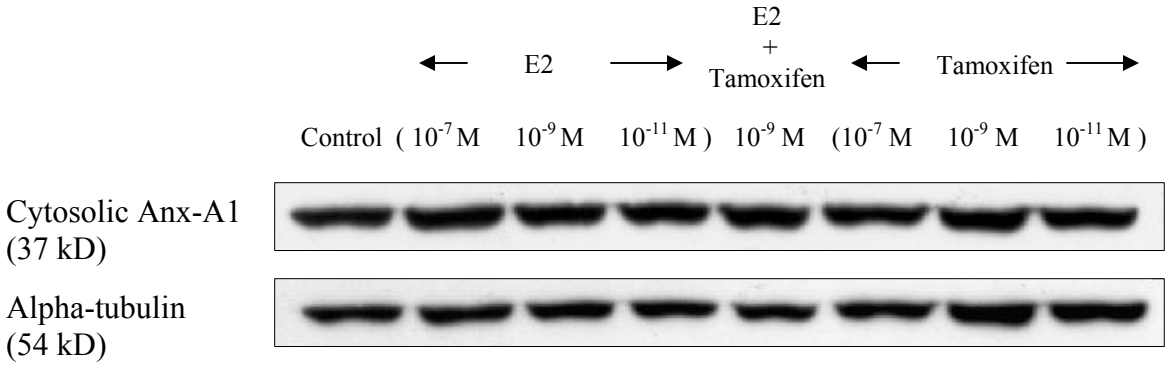


Figure 2: Western blot analysis of Anx-A1 expression after 24 hrs of drug treatment. No difference in expression level was observed. Alpha-tubulin was used as a control to ensure equal loading of protein samples. Data are representative of at least three blots.

48 hrs

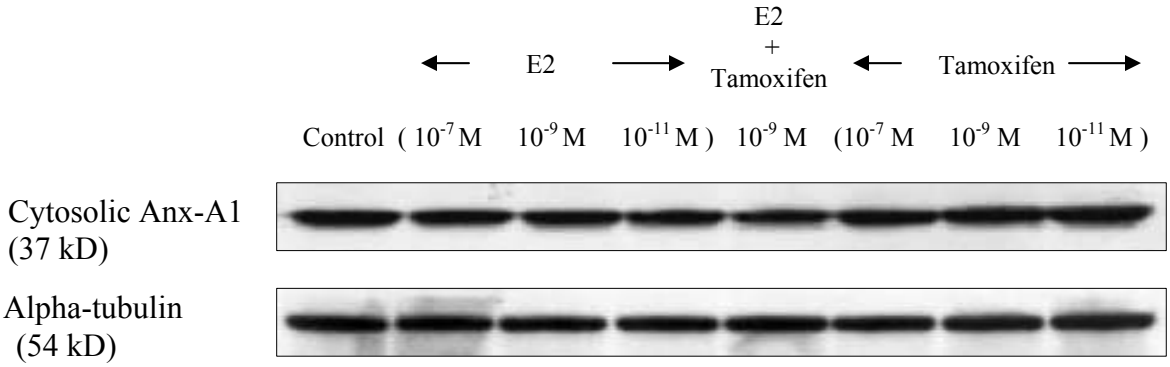
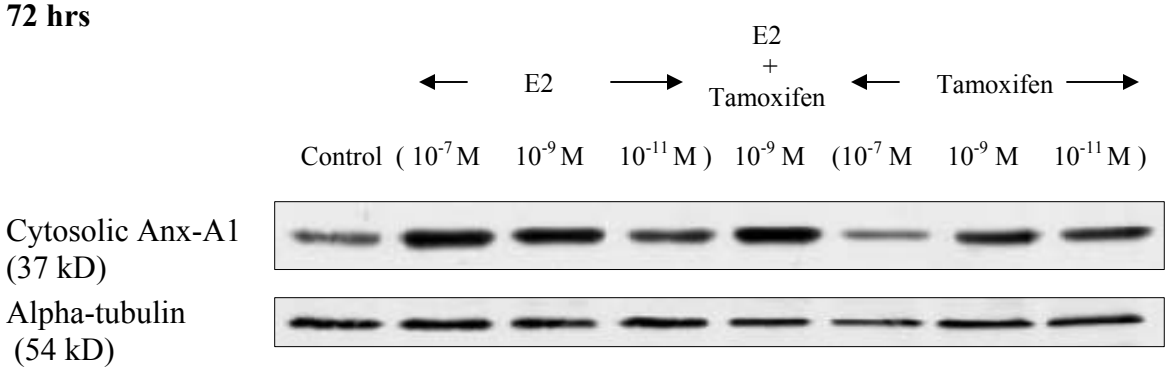


Figure 3: Western blot analysis of Anx-A1 expression after 48 hrs of drug treatment. No difference in expression level was observed. Alpha-tubulin was used as a control to ensure equal loading of protein samples. Data are representative of at least three blots.

A



B

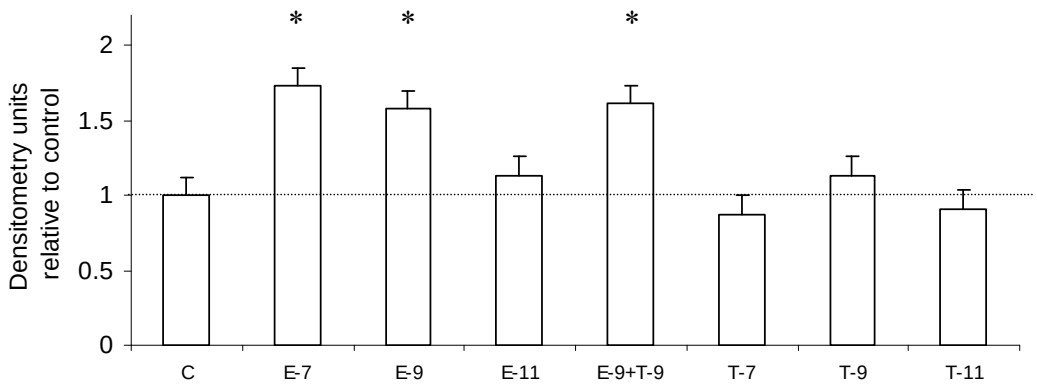


Figure 4: (A) Western blot of cytosolic Anx-A1 levels after 72 hrs of drug treatment. A higher level of Anx-A1 was correlated with a higher dose of E2 treatment. 10^{-9} M of tamoxifen did not inhibit the increase in Anx-A1 level induced by the same dosage of E2. Alpha-tubulin was used as a control to ensure equal loading of protein samples. Data are representative of at least three blots.

(B) Densitometry plot of individual normalized samples against control sample. Values represented are means $\pm$ SEM of three separate experiments. Bars represent standard error bars. Dashed line indicates densitometric intensity of control sample. C: control; E-7, E-9, E-11: E2 at concentrations of 10^{-7} M, 10^{-9} M and 10^{-11} M respectively; T-7, T-9, T-11: tamoxifen at concentrations of 10^{-7} M, 10^{-9} M and 10^{-11} M respectively.
* $P < 0.05$.

4.3 Increase in cytosolic Anx-A1 levels induced by E2 after 72 hrs is not due to translocation

There are two possible reasons which can explain the difference in cytosolic Anx-A1 levels observed after 72 hrs. E2 could have possibly induced Anx-A1 expression in a dose-dependent manner. Alternatively, E2 could have stimulated the translocation of Anx-A1 from a stored pool to the cytosol, thereby leading to an observed increase in cytosolic Anx-A1. We thus examined total Anx-A1 levels in MCF7 cells upon the addition of E2 after 72 hrs.

Western blot analysis of total Anx-A1 extracted from these cells showed that E2-treated MCF7 cells had higher amounts of Anx-A1 as compared to control cells. In addition, the increase in total Anx-A1 induced by E2 was also dose-dependent (Figure 5). Since both cytosolic and total Anx-A1 levels increased in a similar fashion, the increase in cytosolic Anx-A1 observed was not likely due to translocation but an induction of Anx-A1 expression by E2.

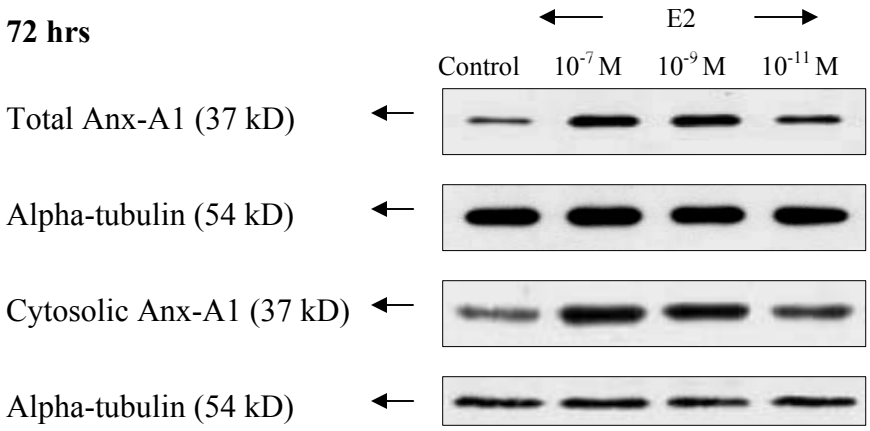


Figure 5: Western blot analysis of cytosolic and total Anx-A1 levels after 72 hrs of drug treatment. Both levels increased in a similar fashion, with a higher level of Anx-A1 being correlated with a higher dose of E2 used. Alpha-tubulin was used as a control to ensure equal loading of protein samples. Data are representative of at least three blots.

4.4 E2 increases Anx-A1 mRNA levels in MCF7 cells after 48 hrs

Since the increase in cytosolic Anx-A1 levels was accompanied by an overall increase in total Anx-A1 expression, it seems likely that E2 can somehow stimulate the expression of Anx-A1 in MCF7 cells. This, coupled with the considerable time lag between drug administration and the observable increase in Anx-A1 levels, suggests that the induction of Anx-A1 expression by E2 is likely to be a result of gene transcription activated either directly or indirectly, by the binding of E2 to its receptor. We thus examined the total mRNA levels of Anx-A1 after 24, 48 and 72 hrs of E2 treatment.

Following RNA extraction, RT-PCR and subsequent DNA gel electrophoresis showed that Anx-A1 mRNA levels increased in a time-dependent manner after treatment with E2 (Figure 6). In addition, while Anx-A1 protein levels showed no observable changes until after 72 hrs of drug treatment, the mRNA levels of Anx-A1 had already increased after 48 hrs at all concentrations of E2 used. This thus supports the hypothesis that E2 induces Anx-A1 expression at the gene transcriptional level.

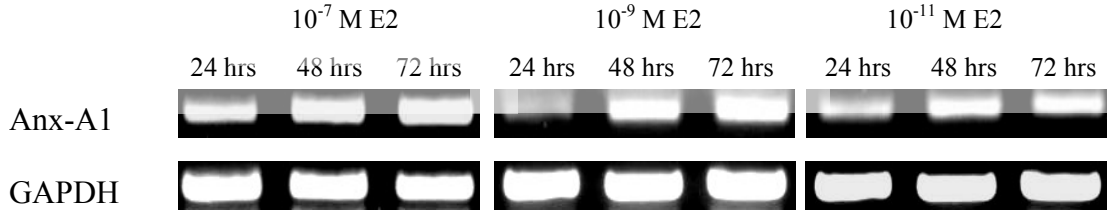


Figure 6: RT-PCR and DNA gel electrophoresis analysis of Anx-A1 mRNA levels after 24, 48 and 72 hrs of E2 treatment. Anx-A1 mRNA levels increased in a time-dependent manner after 48 hrs, even though observable increases in protein levels occurred only after 72 hrs. GAPDH was used as a control to ensure equal loading of samples. Data are representative of at least three analyses.

4.5 Induction of Anx-A1 expression by E2 may occur via action of estrogen receptor-alpha

There are two main estrogen receptors present in MCF7 cells, namely, estrogen receptors (ER) alpha and beta. Although the exact functions and mechanisms of action of the two receptors are not well understood, it has been thought that while the alpha receptors may have positive effects on cell growth and proliferation, the beta receptors may work to counter the effects of ER-alpha by inhibiting their transactivating functions (Macaluso *et al.*, 2006). Since MCF7 cells express both types of receptors, E2 could have induced Anx-A1 up-regulation via either receptor. We hence seek to investigate the possible regulation of Anx-A1 by E2 and/or tamoxifen in MDA-MB-231 breast cancer cells, which express ER-beta, but not ER-alpha.

Western blot analysis of total Anx-A1 levels in MDA-MB-231 cells showed no changes in Anx-A1 expression even after 72 hrs of treatment with E2 and/or tamoxifen (Figures 7, 8 and 9). It is hence possible that E2 induces Anx-A1 expression via the action of ER-alpha.

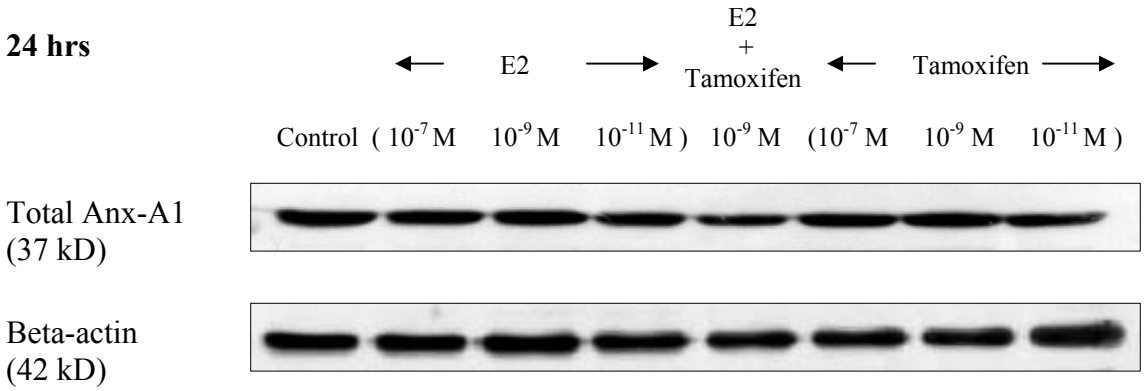


Figure 7: Western blot analysis of Anx-A1 expression after 24 hrs. No difference in expression level was observed. Beta-actin was used as a control to ensure equal loading of protein samples. Data are representative of at least three blots.

48 hrs

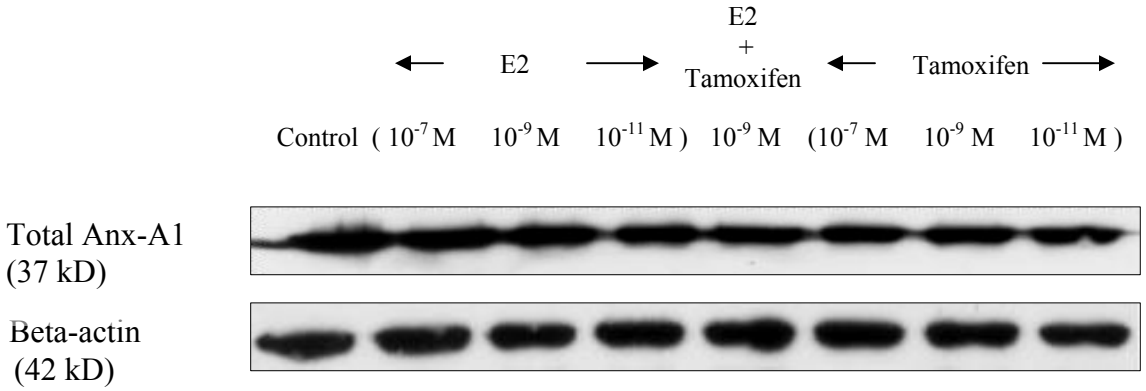


Figure 8: Western blot analysis of Anx-A1 expression after 48 hrs of drug treatment. No difference in expression level was observed. Beta-actin was used as a control to ensure equal loading of protein samples. Data are representative of at least three blots.

72 hrs

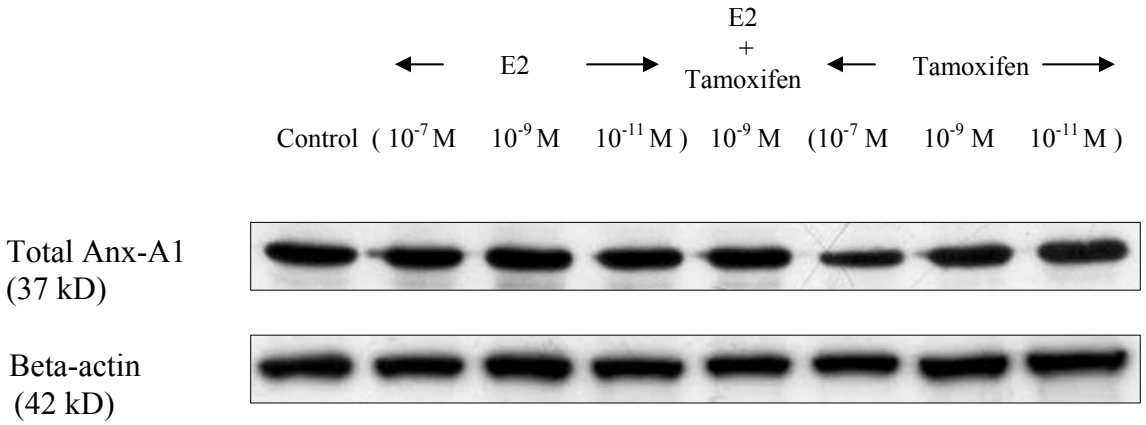


Figure 9: Western blot analysis of Anx-A1 expression after 72 hrs of drug treatment. No difference in expression level was observed. Beta-actin was used as a control to ensure equal loading of protein samples. Data are representative of at least three blots.

4.6 Induction of Anx-A1 expression by E2 is correlated with reduced cell proliferation

Since E2 is a well-established growth stimulatory hormone, we seek to determine if cell proliferation was affected by the experimental conditions. Two different cell proliferation assays, Cell Titer 96® Aq_{ueous} One Solution Cell Proliferation Assay and alamarBlue® Assay, were hence performed. Whereas the former contains a tetrazolium compound that is converted by cells to a toxic product, the alamarBlue® Assay has been demonstrated to be non-toxic to cells and can be used to monitor cell proliferation continuously over a prolonged time period. Hence, while the Cell Titer 96® Aq_{ueous} One Solution Cell Proliferation Assay was used here as an end-point measurement of cell proliferation after the course of drug treatment, the alamarBlue® reagent was added a day after the addition of drug for the continuous monitoring of cell growth over four days.

Both assays showed similar results. Upon 24 or 48 hrs of drug treatment, there were no significant changes in the cell proliferation status (Figure 10). However, following 72 hrs of drug treatment, drug-treated cells showed significant increased cell proliferation, which corresponded with the time of observable Anx-A1 induction. Yet, interestingly, cells treated with the highest dosage of E2 (10^{-7} M) showed the least increase in cell proliferation relative to the control, as compared to other E2 or tamoxifen-treated cells. A high expression of Anx-A1 was thus seemingly correlated with less cell proliferation (Figure 10).

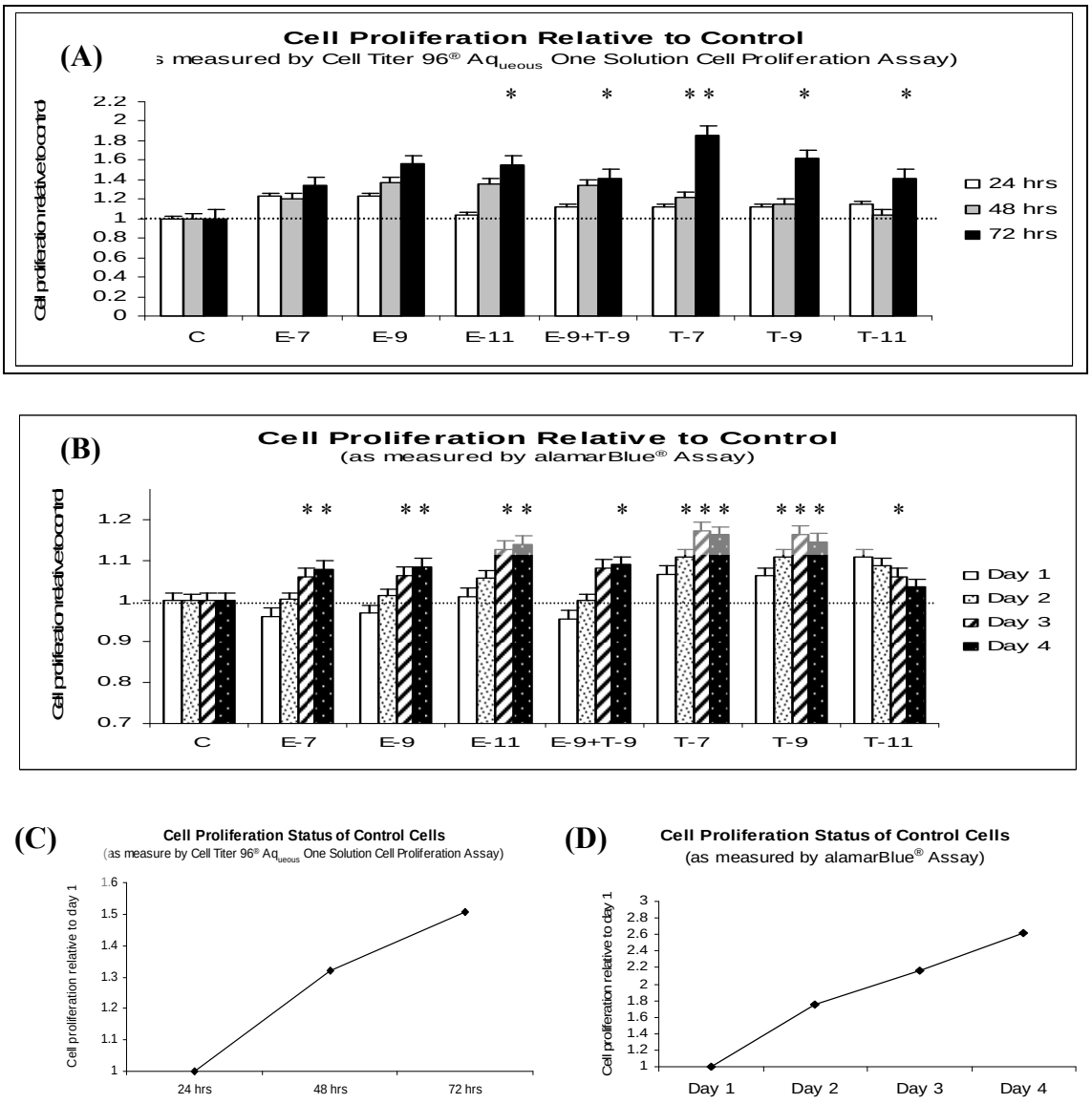


Figure 10: Graphical representations of cell proliferation relative to control cells as measured by (A) Cell Titer 96® Aq_{ueous} One Solution Cell Proliferation Assay and (B) alamarBlue® Assay. Figures (C) and (D) show actual proliferation status of control cells before normalization. Significant cell proliferation was generally observed only after 72 hrs of drug treatment, which corresponded to observable changes in Anx-A1 expression upon drug treatment. Anx-A1 expression and proliferation rates were apparently inversely correlated with each other, and treatment of cells with 10^{-7} M of E2 generally showed the least increase in cell proliferation after 72 hrs.

Data represented are means $\pm$ SEM of at least three separate experiments conducted in triplicates. Bars represent standard error bars. Dashed line indicates relative cell proliferation of control. C: control; E-7, E-9, E-11: E2 at concentrations of 10^{-7} M, 10^{-9} M and 10^{-11} M respectively; T-7, T-9, T-11: tamoxifen at concentrations of 10^{-7} M, 10^{-9} M and 10^{-11} M respectively. * $P < 0.05$

4.7 Differential Anx-A1 expression does not affect apoptosis of MCF7 cells

Because Anx-A1 has been shown to be an endogenous ligand that mediates apoptotic-cell engulfment in a caspase-dependent manner (Arur *et al.*, 2003), the reduced cell proliferation observed in cells treated with 10^{-7} M of E2 could be due to an increase in cell apoptosis mediated by a high expression level of Anx-A1. Annexin V-FITC apoptosis detection staining however showed no significant changes in the percentage of apoptotic cells, even after 72 hrs of drug treatment (Figure 11). The reduced proliferation observed in cells treated with 10^{-7} M of E2 was thus not due to an increase in the percentage of apoptotic cells mediated by a high level of Anx-A1 expression.

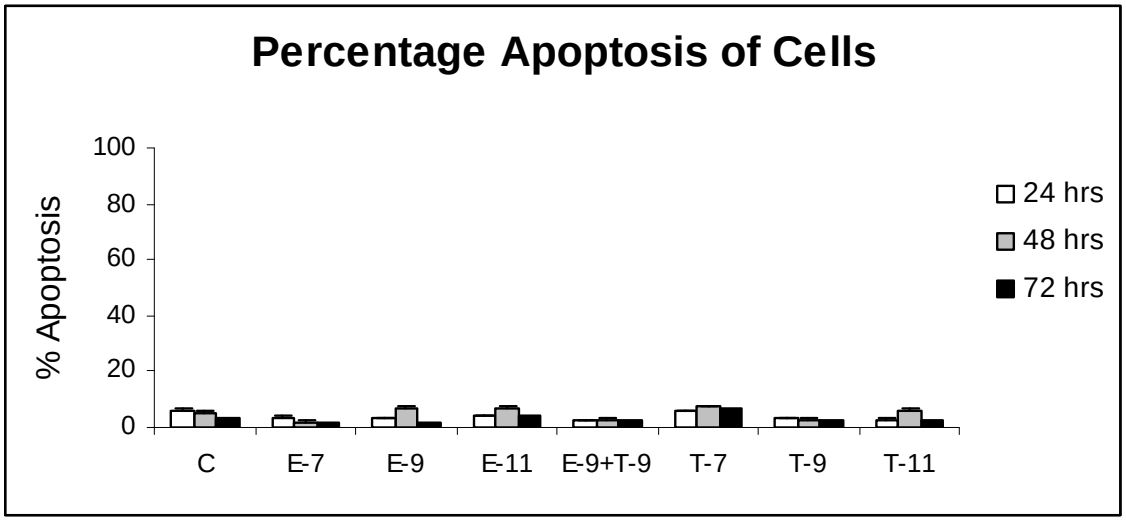


Figure 11: Graphical representation of the percentage of apoptotic cells. No significant changes were observed even after 72 hrs of drug treatment.

Data represented are means ± SEM of at least three separate experiments conducted in triplicates. Bars represent standard error bars. C: control; E-7, E-9, E-11: E2 at concentrations of 10^{-7} M, 10^{-9} M and 10^{-11} M respectively; T-7, T-9, T-11: tamoxifen at concentrations of 10^{-7} M, 10^{-9} M and 10^{-11} M respectively.

4.8 Cell viability is correlated with proliferation status of cells upon drug treatment

Since cells showed increased proliferation rates which were not accompanied by changes in apoptosis, it is logical to deduce that cell viability would be correlated with cell proliferation upon drug treatment, as the number of viable cells will increase as cells divide. Cell viability relative to control cells was thus measured using the MTT assay in order to validate the observations of the cell proliferation and apoptosis assays. Results showed that cell viability was indeed generally consistent with observations from cell proliferation tests (Figure 12).

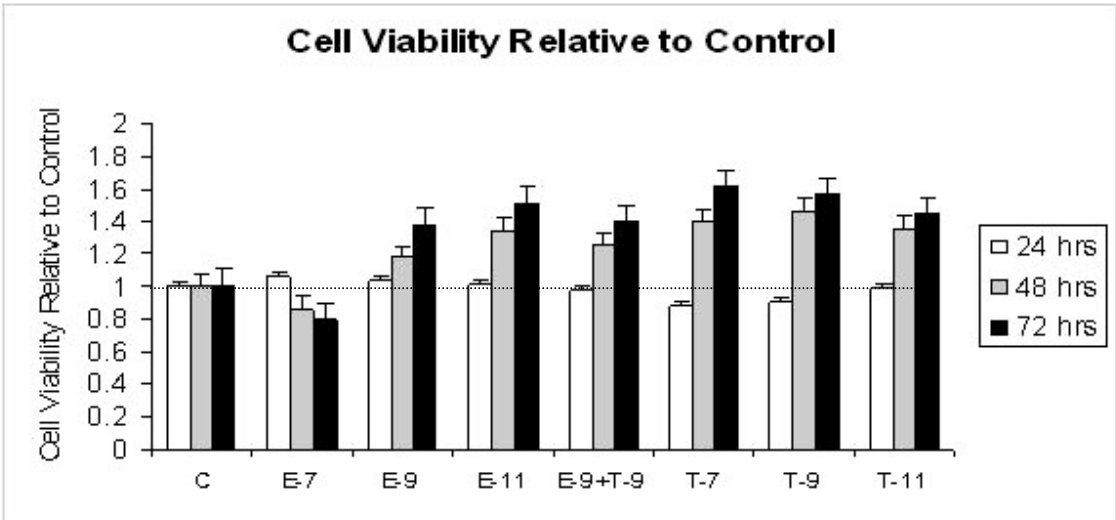


Figure 12: Graphical representation of cell viability relative to control, as measured by MTT cell viability assay. Results were generally consistent with observations from cell proliferation assays.

Data represented are means $\pm$ SEM of at least three separate experiments conducted in triplicates. Bars represent standard error bars. Dashed line indicates relative cell viability of control. C: control; E-7, E-9, E-11: E2 at concentrations of 10^{-7} M, 10^{-9} M and 10^{-11} M respectively; T-7, T-9, T-11: tamoxifen at concentrations of 10^{-7} M, 10^{-9} M and 10^{-11} M respectively.

4.9 The reduced proliferation observed with a high dose of E2 is correlated with a high level of Anx-A1, an ERK activation that was sustained after 72 hrs and an up-regulation of $p21^{waf/cip}$

In 1999, Alldridge *et al.* had reported that while low levels of Anx-A1 expression caused prolonged stimulation of the MAPK pathway and subsequent increased cell proliferation, an over-expression of Anx-A1 led to a *constitutively sustained* stimulation of the same pathway via activation of ERK 1/2 and was accompanied by a *reduction in cell proliferation*, possibly due to an up-regulation of cell-cycle arrest proteins such as $p21^{waf/cip}$ (Alldridge *et al.*, 1999). We thus compared the levels of Anx-A1, $p21^{waf/cip}$ and phosphorylated ERK 1/2 (since ERK 1/2 is activated by phosphorylation at residues Thr 202 and Tyr 204) in the cells after 24 and 72 hrs of drug treatment.

Western blot analysis showed that after 24 hrs of drug treatment, all drug-treated samples had relatively equal amounts of Anx-A1, $p21^{waf/cip}$ and phosphorylated ERK (Figure 13). After 72 hrs however, $p21^{waf/cip}$ expression was up-regulated when cells were treated with higher doses of E2, which correlated well with the lower proliferation observed in these samples (Figure 14). In addition, ERK activation was not sustained after 72 hrs when lower levels of E2 that could stimulate higher cell proliferation were used (Figure 14). Hence, it would appear that in line with Alldridge *et al.*'s observations, a constitutively sustained ERK activation and an up-regulation of Anx-A1 may indeed have led to the reduced proliferation observed when cells were treated with high E2 doses.

Tamoxifen, on the other hand, appears to act as an agonist in the context of our experiments and in contrast to E2, could induce cell proliferation despite an ERK activation that was sustained after 72 hrs.

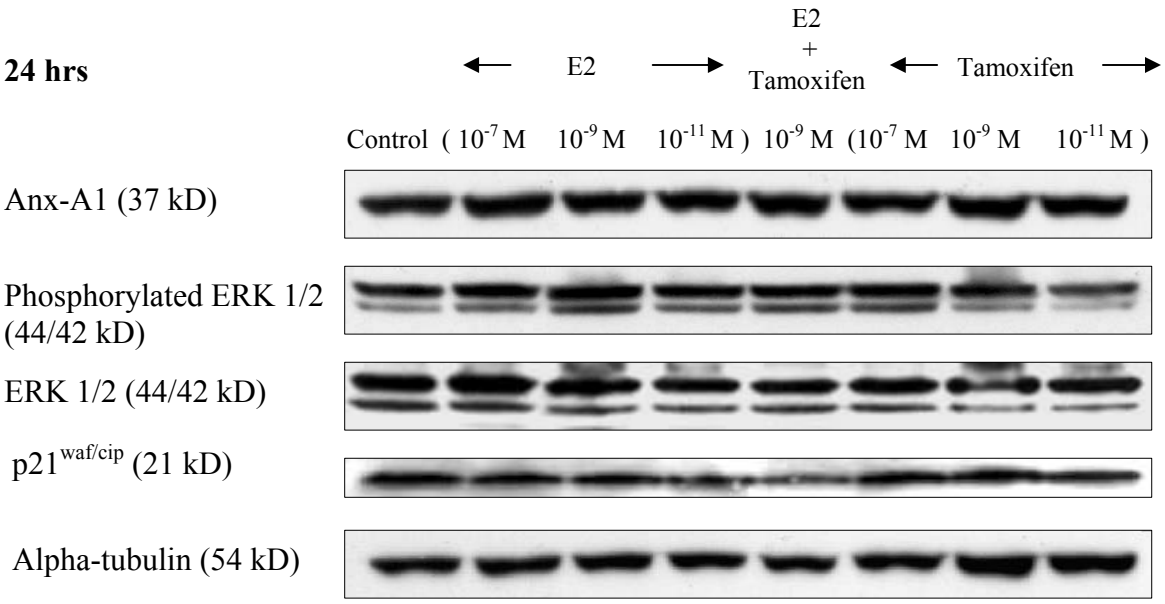


Figure 13: Western blot analysis of ERK activation, $p21^{waf/cip}$ and Anx-A1 expression levels after 24 hrs of drug treatment. No significant differences were observed. Alpha-tubulin and ERK 1/2 were used as controls to ensure equal loading of protein samples. Data are representative of at least three blots.

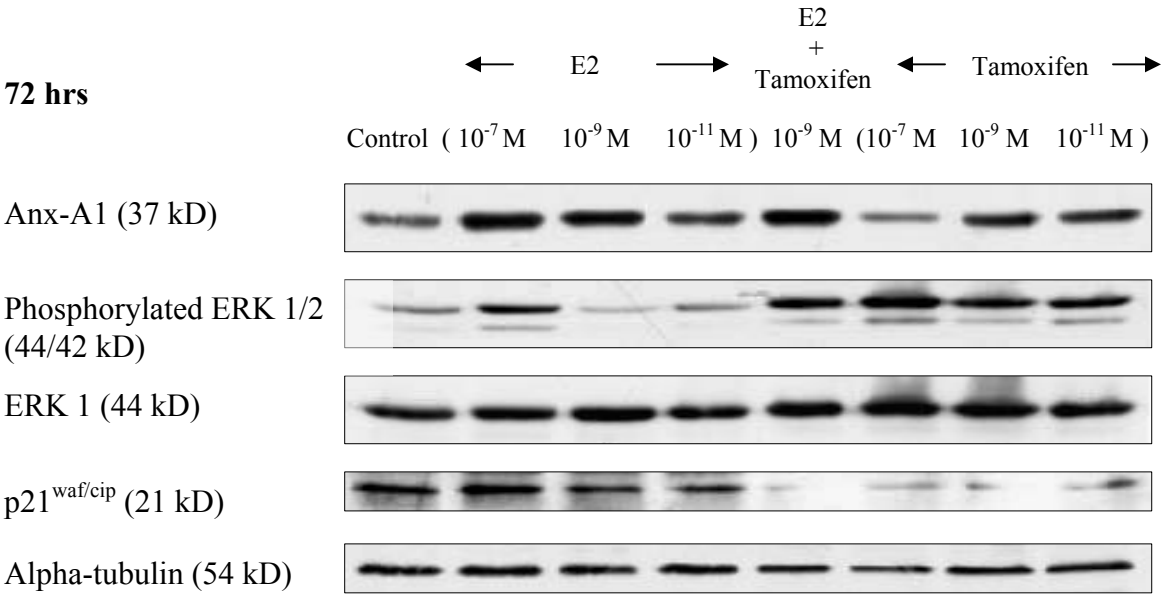


Figure 14: Western blot analysis of ERK activation, $p21^{waf/cip}$ and Anx-A1 expression levels after 72 hrs of drug treatment. Sustained ERK activation, high Anx-A1 and $p21^{waf/cip}$ levels were observed when cells were treated with a high dose of E2. This was in turn correlated with lower cell proliferation. In contrast, tamoxifen could induce cell proliferation despite a constitutive ERK activation. Alpha-tubulin and ERK 1 were used as controls to ensure equal loading of protein samples. Data are representative of at least three blots.

4.10 Anx-A1 inhibits cell proliferation, possibly via a constitutively sustained activation of ERK in MCF7 cells

To investigate if the expression of Anx-A1 in MCF7 cells was induced by E2 for a specific reason, or if Anx-A1 was merely up-regulated as a by-stander gene, transfection studies were first performed to elucidate the physiological functions of Anx-A1 in these cells.

Gene expression of Anx-A1 was silenced via transfection of MCF7 cells with Dharmacon *SMART*pool® siRNA, which utilizes a pool of four *SMART*selection™ designed siRNA duplexes and could reduce the expression of Anx-A1 by approximately 60% even after five days (Figure 15). Interestingly, while Anx-A1-knockdown cells had less ERK activation even after five days, they typically demonstrated a 20% increase in cell proliferation rates (Figures 15 and 16). In contrast, when gene expression of Anx-A1 was increased via transfection of MCF7 cells with a plasmid expressing ANX-A1 fused with a V5 tag, cell proliferation rates were reduced, despite an enhanced ERK activation that was sustained even after five days (Figures 17 and 18).

Such an observation is thus in line with the fact that while ERK is conventionally seen as a positive regulator of pro-proliferative processes, a sustained ERK activation may lead to anti-proliferative effects such as cell cycle arrest (Tang *et al.*, 2002) and differentiation (Wang *et al*., 2003). At the same time, it also correlates well with a study which showed that Anx-A1 exerts anti-proliferative effects via sustained activation of ERK in macrophages and human embryonic kidney cells (Alldridge and Bryant, 2003). Anx-A1 is hence, able to inhibit cell proliferation in MCF7 cells, and may achieve this via a sustained over-activation of ERK.

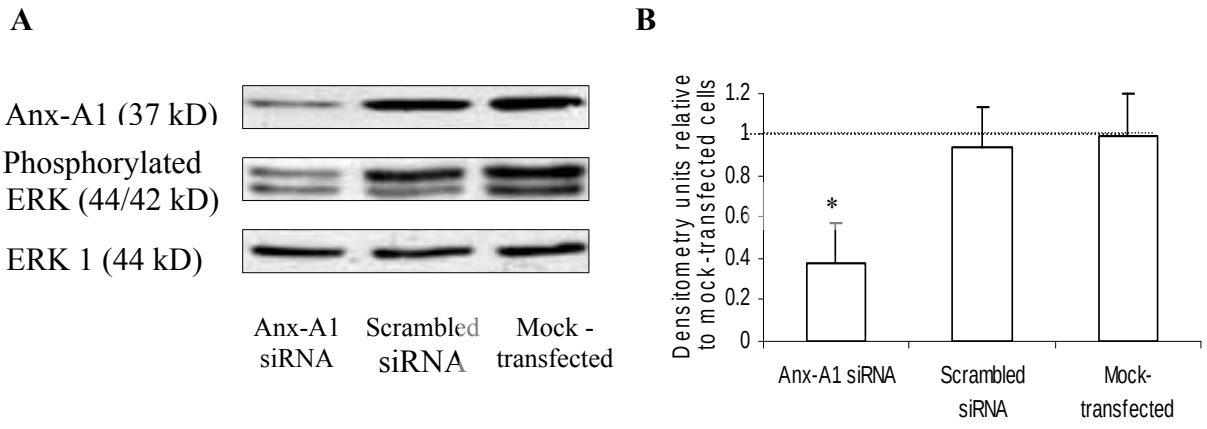


Figure 15: (A) Western blot of total Anx-A1 levels five days after transfection with Dharmacon *SMART*pool® siRNA. ERK 1 was used as a control to ensure equal loading of protein samples. Data are representative of at least three blots. Anx-A1 siRNA and Scrambled siRNA: transfection with Anx-A1 and scrambled siRNA respectively; Mock-transfected: transfection with oligofectamine alone. (B) Densitometry plot of normalized samples against mock-transfected cells. Values represented are means ± SEM of three separate experiments. Bars represent standard error bars. Dashed line indicates densitometric intensity of mock-transfected cells. * $P < 0.05$.

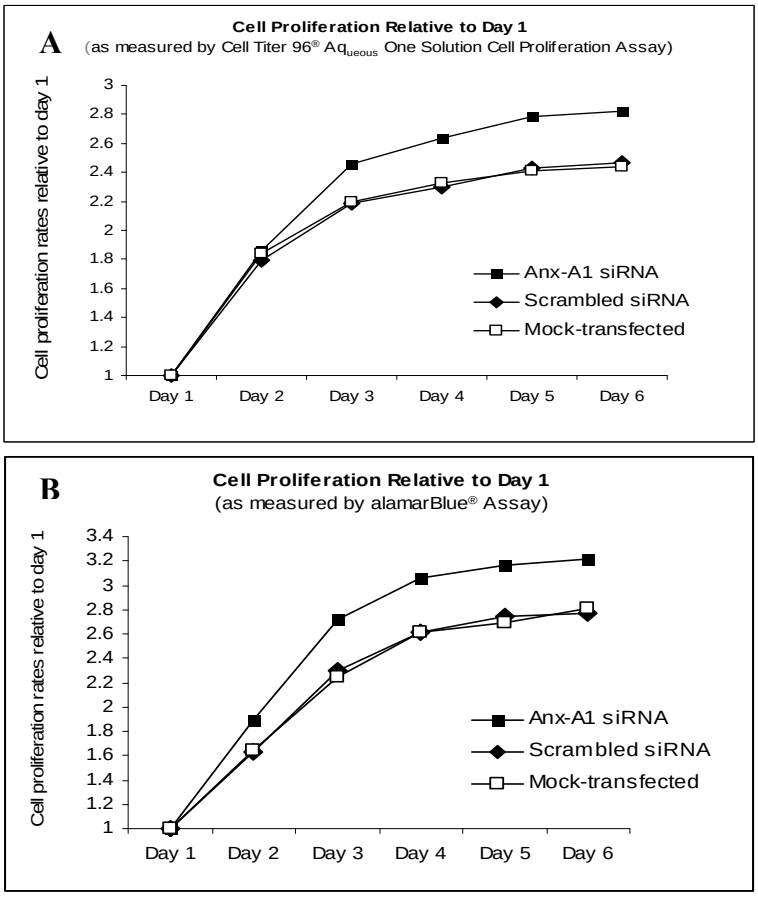


Figure 16: Graphical representations of cell proliferation relative to day 1 of Anx-A1 siRNA, scrambled siRNA and mock-transfected cells, as measured by (A) Cell Titer 96® Aq_{ueous} One Solution Cell Proliferation Assay and (B) alamarBlue® Assay.

Both assays showed similar results - cells transfected with Anx-A1 siRNA typically showed approximately 20% increase in cell proliferation rates, as compared to scrambled siRNA or mock-transfected cells. Values represented are means ± SEM of at least three separate experiments conducted. Anova p-values: (A) 0.00655 and (B) 0.0149.

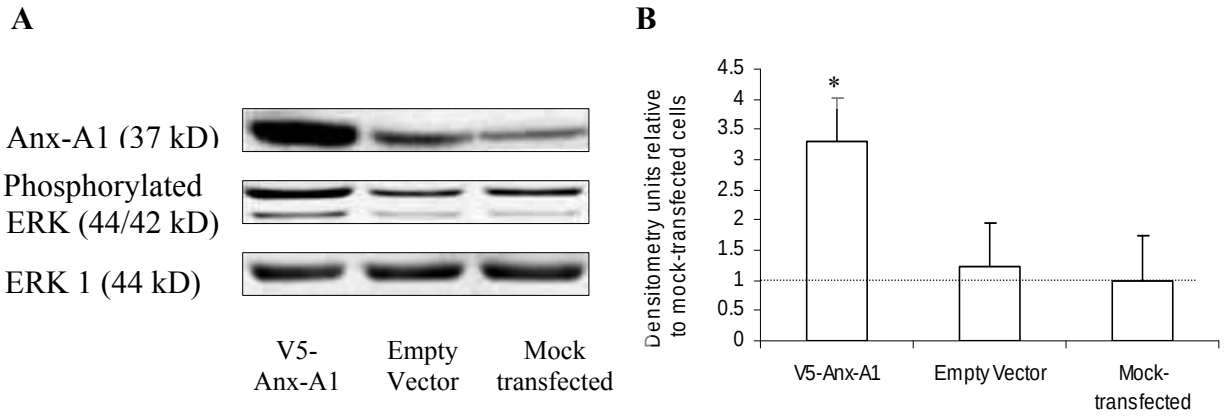


Figure 17: (A) Western blot of total Anx-A1 levels five days after transfection with a DNA plasmid that expresses Anx-A1 fused with a V5 tag. ERK 1 was used as a control to ensure equal loading of protein samples. Data are representative of at least three blots. V5-Anx-A1 and Empty Vector: transfection with Anx-A1 and empty vector respectively; Mock-transfected: transfection with superfectamine alone. (B) Densitometry plot of normalized samples against mock-transfected cells. Values represented are means ± SEM of three separate experiments. Bars represent standard error bars. Dashed line indicates densitometric intensity of mock-transfected cells. * $P < 0.05$.

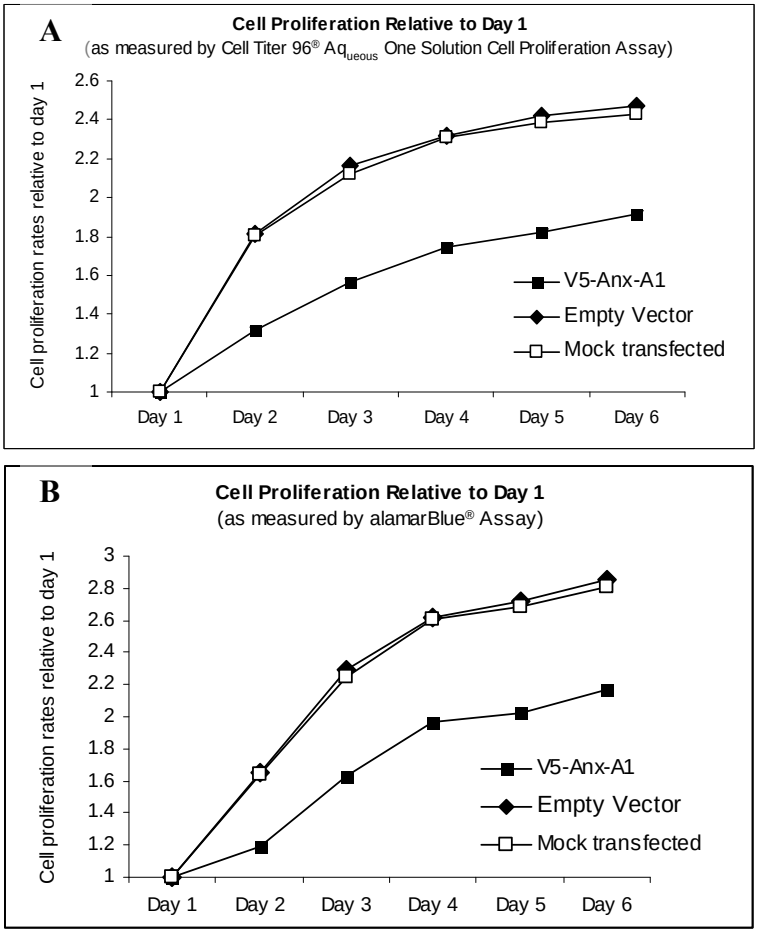


Figure 18: Graphical representations of cell proliferation relative to day 1 of V5-Anx-A1, empty vector and mock-transfected cells, as measured by (A) Cell Titer 96® Aq_{ueous} One Solution Cell Proliferation Assay and (B) alamarBlue® Assay.

Both assays showed similar results - cells transfected with V5-Anx-A1 typically showed approximately 20-30% decrease in cell proliferation rates, as compared to empty vector or mock-transfected cells. Values represented are means ± SEM of at least three separate experiments conducted. Anova p-values: (A) 0.00627 and (B) 0.006947.

4.11 Anx-A1 is required to sustain the activation of ERK observed in MCF7 cells exposed to high E2 doses after 72 hrs

As our transfection studies have shown that silencing of Anx-A1 expression led to reduced ERK activation and that over-expression of Anx-A1 resulted in a corresponding increase in ERK activation even after five days, it can be postulated that Anx-A1 is able to activate ERK either directly, or indirectly. In addition, this sustained activation of ERK by Anx-A1 is likely to be responsible for Anx-A1's anti-proliferative effects on cells as Alldridge and Bryant had demonstrated previously that Anx-A1 may inhibit cell proliferation via an ERK-mediated disruption of the actin cytoskeleton and ablation of cyclin D1 expression in macrophages and human embryonic kidney cells (Alldridge and Bryant, 2003). We hence seek to determine if the induction of Anx-A1 by E2 is responsible, at least in part, for the sustained ERK activation observed when cells were treated with high E2 doses for 72 hrs.

Western blot analysis showed that similar to un-transfected cells, MCF7 cells transfected with scrambled siRNA showed high Anx-A1 expression and ERK activation after 72 hrs when treated with high E2 doses. In contrast, cells transfected with Anx-A1 siRNA were unable to induce significant Anx-A1 expression and were consequently, unable to induce high ERK activation after 72 hrs when treated with high E2 doses (Figure 19). Anx-A1 is thus required to sustain the activation of ERK observed in MCF7 cells exposed to high E2 concentrations.

72 hrs

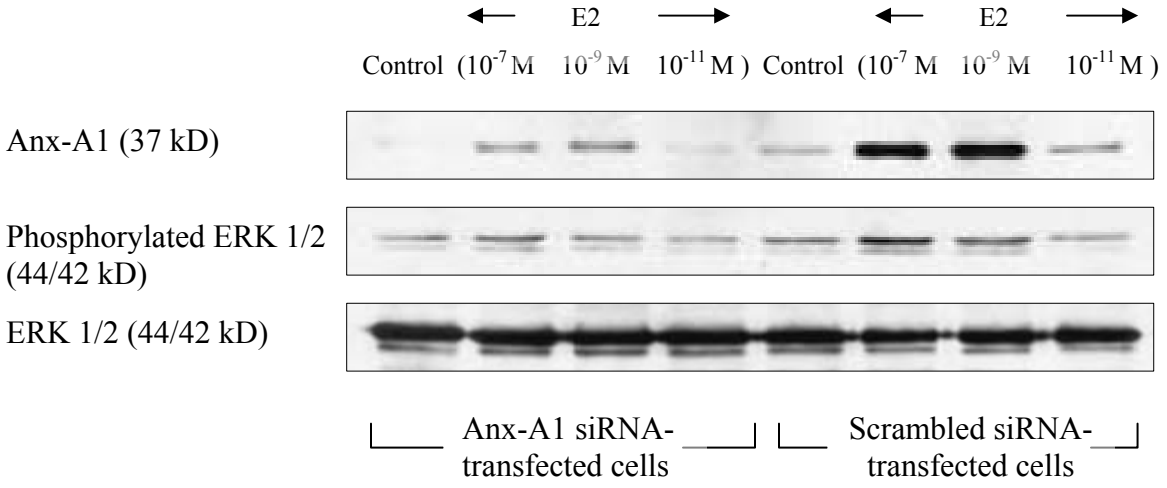


Figure 19: Western blot analysis of ERK activation after Anx-A1/ scrambled siRNA-transfected cells were treated with E2 for 72 hrs. Scrambled siRNA-transfected cells showed a dose-dependent increase of Anx-A1 after 72 hrs, and a higher ERK activation was also observed after 72 hrs of treatment with higher E2 doses. In contrast, Anx-A1 siRNA transfected cells were unable to induce significant Anx-A1 expression and ERK activation. ERK 1/2 was used as a control to ensure equal loading of protein samples. Data are representative of two blots.

4.12 Anx-A1 plays a direct role in the reduced cell proliferation rates observed when MCF7 cells were exposed to higher E2 concentrations

Since Anx-A1 has direct anti-proliferative functions in MCF7 cells under normal experimental conditions, the up-regulation of Anx-A1 due to prolonged treatment of cells with high E2 doses could be responsible for the reduced growth rates observed for these cells. To confirm the direct role of Anx-A1 in the lower cell proliferation rates observed when cells were exposed to high E2 concentrations over a prolonged period of time (after 72 hrs), cells were first transfected with either Anx-A1 siRNA or scrambled siRNA, before being treated with E2 and/or tamoxifen. Cell proliferation rates were then measured using the Cell Titer 96® Aq_{ueous} One Solution Cell Proliferation Assay and alamarBlue® Assay.

Cells transfected with scrambled siRNA showed proliferation rates that were similar to un-transfected drug-treated cells – cells treated with 10^{-7} M of E2, 10^{-9} M of E2 and 10^{-9} M of both tamoxifen and E2 typically showed the least increase in cell proliferation relative to the control (Figures 20B and 21B). In contrast, MCF7 cells with Anx-A1 expression suppressed via transfection with Anx-A1 siRNA, now demonstrated the highest proliferation rates when treated with high E2 doses (Figures 20A and 21A). Since suppression of Anx-A1 expression was able to remove the inhibition of cell growth induced by prolonged exposure to high levels of E2, it thus follows that Anx-A1 plays a direct role in the anti-proliferative effects brought about by high concentrations of E2.

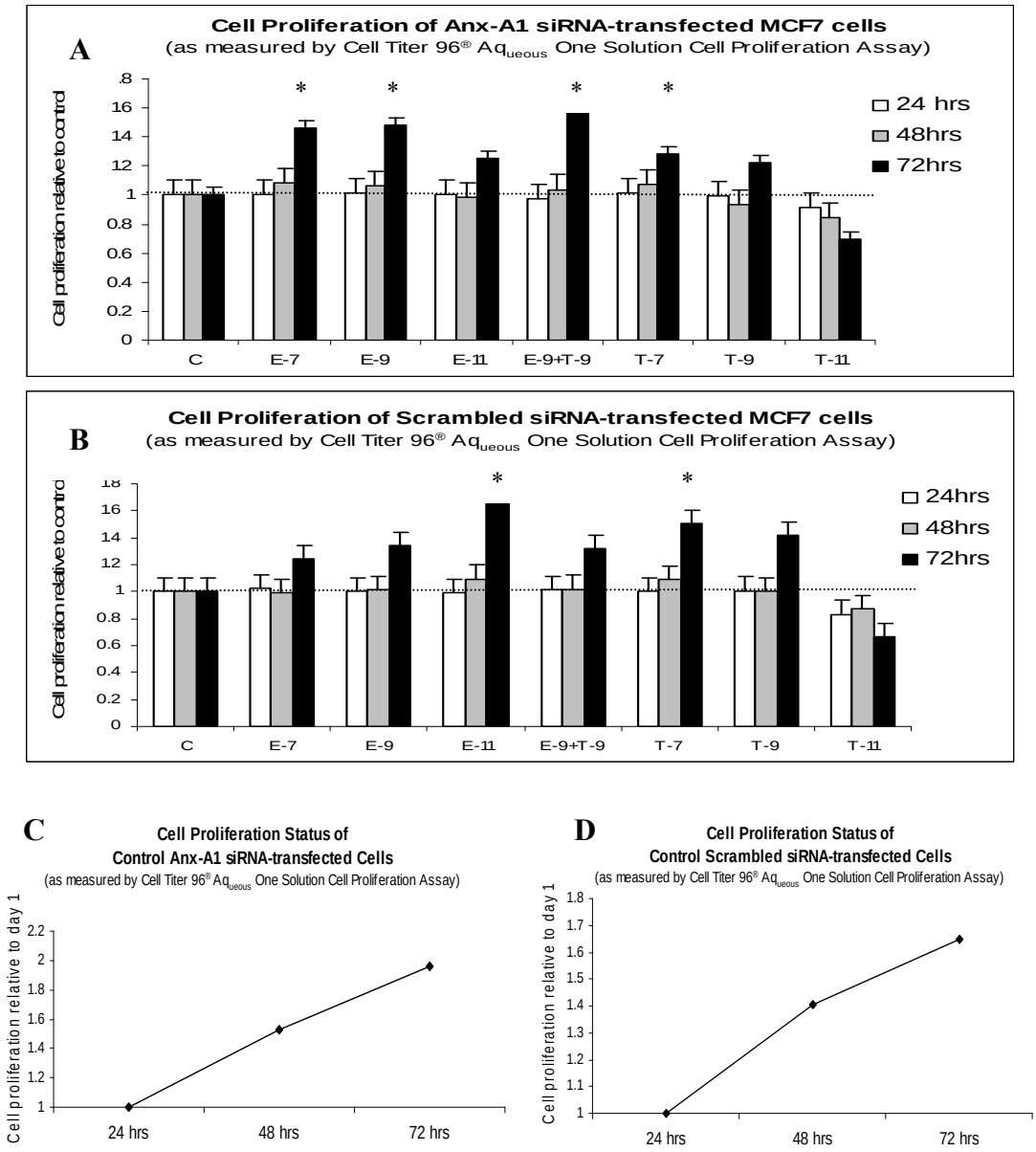


Figure 20: Graphical representations of cell proliferation relative to control of (A) Anx-A1 siRNA-transfected and (B) scrambled siRNA-transfected cells as measured by Cell Titer 96® Aq_{ueous} One Solution Cell Proliferation Assay. Figures (C) and (D) show actual proliferation status of control cells before normalization. Silencing of Anx-A1 expression was able to remove the inhibition of cell growth induced by prolonged exposure to 10^{-7} M of E2, 10^{-9} M of E2 and 10^{-9} M of both tamoxifen and E2.

Data represented are means $\pm$ SEM of at least three separate experiments conducted in triplicates. Bars represent standard error bars. Dashed line indicates relative cell proliferation of control. C: control; E-7, E-9, E-11: E2 at concentrations of 10^{-7} M, 10^{-9} M and 10^{-11} M respectively; T-7, T-9, T-11: tamoxifen at concentrations of 10^{-7} M, 10^{-9} M and 10^{-11} M respectively. * $P < 0.05$

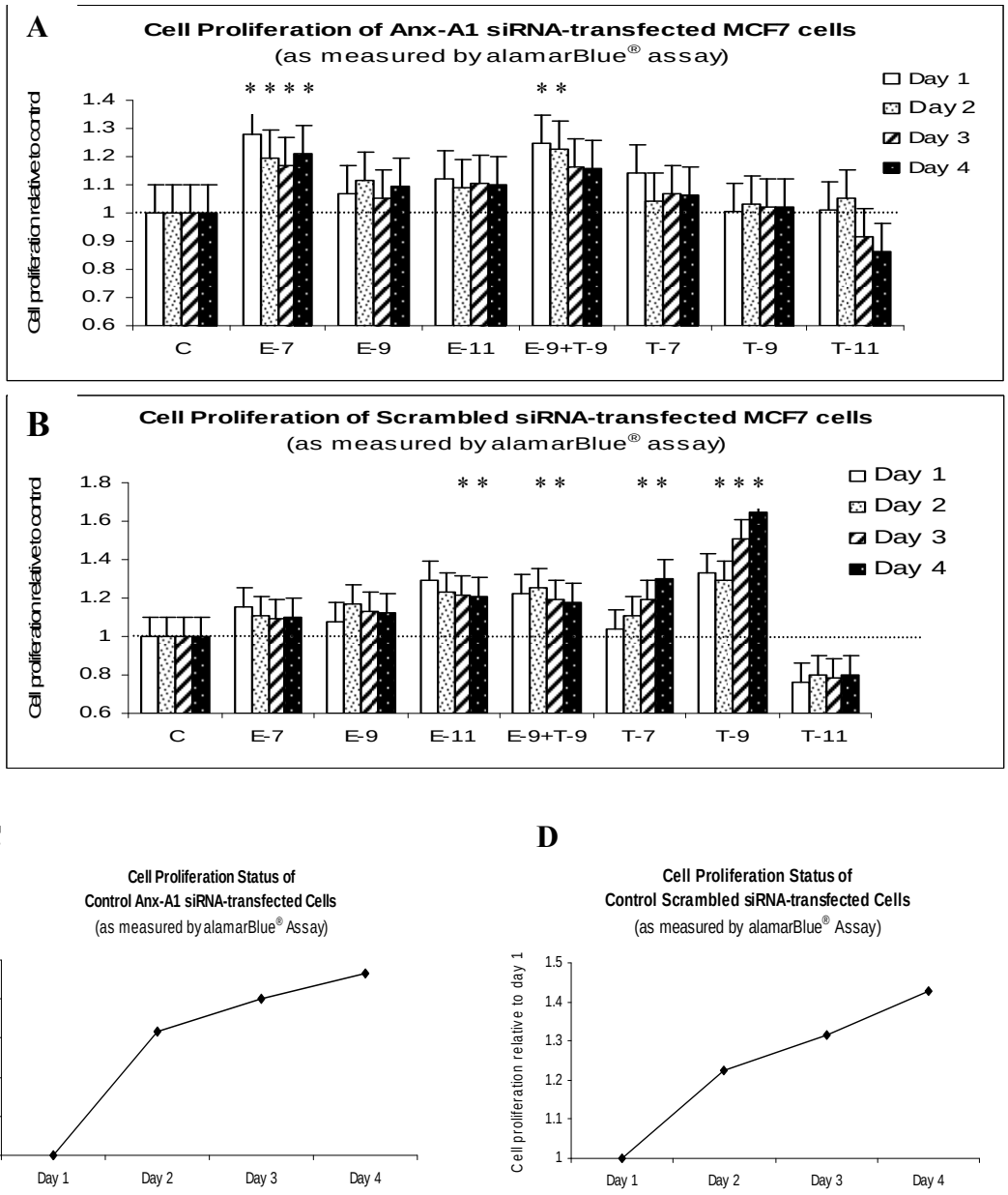


Figure 21: Graphical representations of cell proliferation relative to control of (A) Anx-A1 siRNA-transfected and (B) scrambled siRNA-transfected cells as measured by alamarBlue® Assay. Figures (C) and (D) show actual proliferation status of control cells before normalization. Silencing of Anx-A1 expression was able to remove the inhibition of cell growth induced by prolonged exposure to 10^{-7} M of E2, 10^{-9} M of E2 and 10^{-9} M of both tamoxifen and E2.

Data represented are means $\pm$ SEM of at least three separate experiments conducted in triplicates. Bars represent standard error bars. Dashed line indicates relative cell proliferation of control. C: control; E-7, E-9, E-11: E2 at concentrations of 10^{-7} M, 10^{-9} M and 10^{-11} M respectively; T-7, T-9, T-11: tamoxifen at concentrations of 10^{-7} M, 10^{-9} M and 10^{-11} M respectively. * $P < 0.05$

4.13 The sustained ERK activation following prolonged exposure to high E2 doses does not provide the direct anti-proliferative signal to induce Anx-A1 expression

Because we have shown that Anx-A1 has direct anti-proliferative effects on MCF7 cells, it is not likely that E2, being a well-established potent pro-proliferative hormone, would induce the up-regulation of Anx-A1 for its own purposes. Instead, it is more plausible that prolonged exposure to high levels of E2 had over-stimulated certain cell-signaling pathways and that as a counter-mechanism, Anx-A1 was up-regulated to inhibit cell proliferation.

As mentioned previously, a constitutive activation of ERK may lead to anti-proliferative effects such as cell cycle arrest and differentiation. We would hence, like to investigate if the direct signal for Anx-A1 up-regulation was in fact, an over-activation of ERK induced by high E2 doses in the first place. If the direct anti-proliferative signal for Anx-A1 expression indeed requires a prolonged over-activation of ERK, it would mean that Anx-A1 and ERK may partake in a feedback mechanism, whereby an over-activation of ERK leads to the up-regulation of Anx-A1, which is in turn, required to maintain this high ERK activation constitutively to repress cell proliferation (since we had previously established that the induction of Anx-A1 by E2 is required for the sustenance of a prolonged ERK activation after 72 hrs and an inhibition of cell proliferation). We thus examined Anx-A1 expression levels in MCF7 cells which were pre-treated with U0126 (a MEK inhibitor) prior to the addition of E2 after 72 hrs.

Western blot analysis showed that inhibition of ERK activation by U0126 did not abolish the up-regulation of Anx-A1 as induced by E2 after 72 hrs (Figures 22 and 23). Hence, while it is still plausible that an over-activation of cell-signaling pathways by E2 is

the direct cause of Anx-A1 induction, the over-activation of ERK does not provide the direct signal to increase Anx-A1 expression in the context of this study.

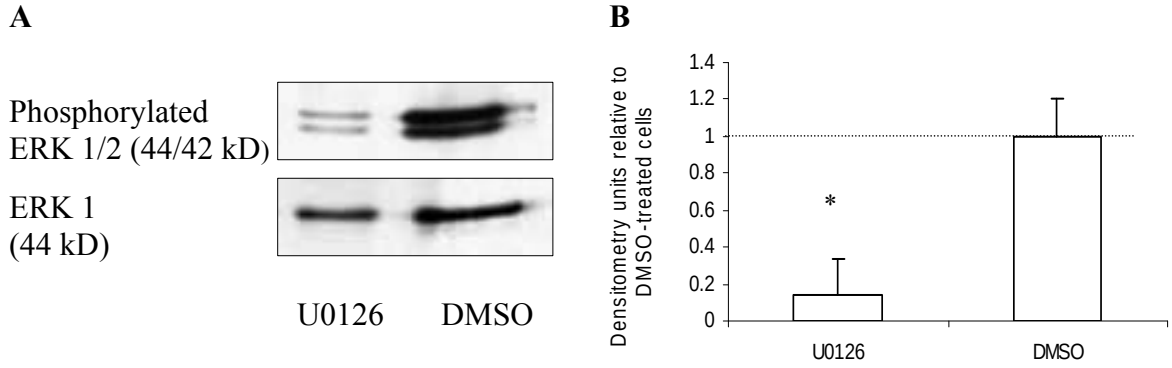


Figure 22: (A) Western blot of ERK activation levels. Cells were pre-treated with either U0126 or DMSO (as control) before the degree of inhibition of ERK activation was measured after 72 hrs. ERK 1 was used as a control to ensure equal loading of protein samples. Data are representative of three blots. (B) Densitometry plot showing the level of ERK activation in U0126 and DMSO-treated cells. Data represented are means $\pm$ SEM of three separate experiments. Bars represent standard error bars. Dashed line indicates densitometric intensity of DMSO-treated control cells. * $P < 0.05$.

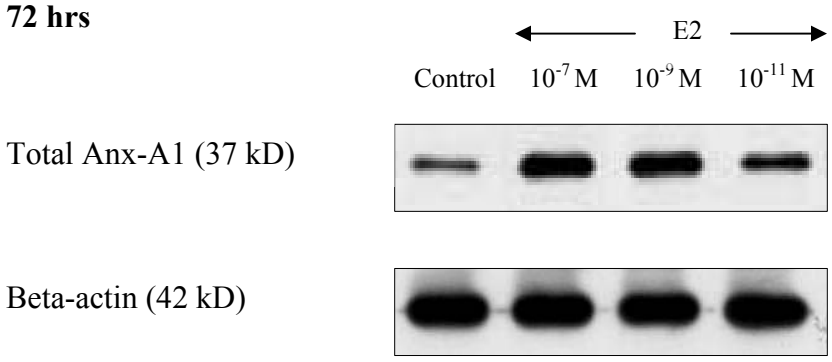


Figure 23: Western blot analysis of Anx-A1 expression after 72 hrs of drug treatment. Cells were pre-treated with U0126 to inhibit ERK activation prior to the addition of drugs. ERK inhibition had no apparent effects on the induction of Anx-A1 expression by E2. Beta-actin was used as a control to ensure equal loading of protein samples. Data are representative of three blots.

5. DISCUSSION

In this study, we have demonstrated for the first time that Anx-A1 can be induced by E2 in MCF7 breast cancer cells and that this induction appears to be a delayed response occurring over a period of more than 48 hrs. Due to the considerable time lag between drug administration and the observable increase in Anx-A1 expression, as well as the fact that Anx-A1's mRNA levels were increased following treatment of the cells with E2, the induction of Anx-A1 is likely to be a result of gene transcription activated either directly or indirectly, by the binding of E2 to its receptor. In addition, as mentioned previously, it is highly plausible that the up-regulation of Anx-A1 is due to the action of E2 on ER-alpha, and not ER-beta, since treatment of MDA-MB-231 cells (which express only ER-beta, and not ER-alpha) with E2 had no apparent effects on Anx-A1 expression whatsoever. Although the role of ER-beta remains elusive (Gustafsson and Warner, 2000), some studies have suggested that while actions of E2 via ER-alpha are typically pro-proliferative, the effects of E2 on ER-beta are in contrast, anti-proliferative and tumor suppressive (Paruthiyil S. *et al*., 2004). Since Anx-A1 was likely up-regulated as a result of E2 on ER-alpha, the expression of Anx-A1 may have been induced as a result of E2's pro-proliferative effects on cells.

It should also be noted that whilst tamoxifen by itself, did not exert observable affects on Anx-A1 expression, treatment of the cells with 10^{-9} M of both tamoxifen and E2 led to an up-regulation of Anx-A1 expression. Tamoxifen, a drug used often as an anti-estrogen in breast cancer treatment, was thus unable to inhibit the induction of Anx-A1 expression by E2. This is not however, a surprising observation since it is a well-

known fact that tamoxifen can act either as an agonist or an antagonist of E2, depending on factors such as the cellular context and the dosage used. The agonistic effects of tamoxifen would be later confirmed by the fact that tamoxifen could induce proliferation of MCF7 cells in our study.

In 2001, Castro-Caldas *et al*. had reported that E2 can promote the synthesis and secretion of Anx-A1 in the human CCRF-CEM acute lymphoblastic leukemia cell line within 30 min, and that this may account for E2's reported anti-inflammatory effects on lymphocytes (Castro-Caldas *et al*., 2001). The considerable difference in terms of the time required to observe Anx-A1 up-regulation following E2 treatment between this study and Castro-Caldas's report may be due to the usage of different cell lines. Since Anx-A1 has been implicated in various different cellular functions, it is possible that the induction of Anx-A1 by E2 serves different purposes in different cells and thus, E2 may require different amounts of time to increase Anx-A1 expression in lymphoid and mammary cells. In addition, the concentrations of E2 used in Castro-Caldas's *et al.* study were in the micromolar range, whereas nanomolar concentrations of E2 were used in our study. Differences in concentrations of E2 used may hence also account for the disparity between our results and Castro-Caldas's study.

The induction of Anx-A1 in MCF7 cells by E2 is an interesting observation, not only because of its possibly significant role in breast tumorigenesis, but also because it was reported previously that only gluccocorticoids, but not the chemically similar sex hormones, could induce Anx-A1 expression (Ahluwalia *et al.*, 1996). While it is possible

that these differing results were due to the usage of different cell lines, the apparent lack of Anx-A1 induction by sex hormones in previous studies may also be due to the fact that endogenous steroids present in cell culture media had already induced a high expression level of Anx-A1 in cells, which may then mask any further induction of Anx-A1 expression by estrogens (or androgens). The latter explanation is highly plausible since other groups had noted that the presence of endogenous gluccocorticoids in serum induced large amounts of Anx-A1 which in turn, masked any subsequent gluccocorticoid-induction of Anx-A1 (Croxtall and Flower, 1992). Incubation of cells in serum-free or charcoal-treated cell culture medium may thus be an important precondition necessary to observe changes in cellular Anx-A1 expression.

The regulation of Anx-A1 expression by E2 can account for our observation that Anx-A1 was differentially expressed in the normal and tumorigenic breast tissues used in our study. Breast cancers can be classified as estrogen receptor-positive or negative, depending on whether the breast cancer cells contain estrogen receptors or not. If the induction of Anx-A1 by E2 can be confirmed in an *in vivo* model, it is possible that the tumor cells with higher Anx-A1 expression were estrogen receptor positive and that the patient had a physiologically high concentration of the hormone within the body. Within our study, data information with regards to the estrogen level and class of breast cancer of the patients was not given. It may thus be interesting if possible, to correlate the Anx-A1 expression in breast tumorigenic cells with such data.

In cell proliferation assays conducted within this study, it was observed that tamoxifen induced cell proliferation at a rate similar to that of low doses of estrogen. Tamoxifen works as an anti-cancer drug by competing with natural estrogens within our body for estrogen receptors. Because it acts as a weak estrogen, by binding to estrogen receptors, tamoxifen thus has seemingly anti-estrogenic effects, simply because it prevents beta-estradiol, the strongest natural estrogen within the body from acting. Yet, it should be remembered that tamoxifen, being an estrogen analogue, has mild estrogenic effects as well. In our study, cells were pre-incubated with charcoal-treated cell culture medium to remove the presence of endogenous steroid hormones. Because endogenous estrogen within the medium had been removed, it should not be unexpected that tamoxifen would exhibit its inherent mildly estrogenic effects. The observed increase in cell proliferation induced by tamoxifen is hence, not contradictory to the usage of tamoxifen as an anti-estrogen in cancer treatment.

However, the consistent reduction in proliferation when cells were treated with 10^{-7} M of E2 as compared to other drug-treated cells was a surprising result. E2, being a growth hormone, is well known for its growth-stimulatory effects, especially in breast tissues and a higher dose of E2 should therefore theoretically, induce a higher proliferation rate in MCF7 cells. Subsequent western blot analyses showed that treatment of the cells with 10^{-7} M of E2 had led to an ERK activation that was sustained even after 72 hrs, unlike 10^{-9} M and 10^{-11} M which resulted in an ERK activation that was not sustained after 72 hrs. Since E2 has been shown to be able to activate the MAPK pathway in other studies as well (Improta-Brears *et al.*, 1999) and that a *constitutive* activation of

the MAPK pathway often leads to anti-proliferative signals being generated to restrict cell proliferation (Tang *at al.*, 2002 and Wang *et al.*, 2003), it is likely that prolonged exposure to high levels of E2 had induced a constitutive over-activation of ERK that had in turn, resulted in anti-proliferative effects.

As at this point, there are three possible hypotheses which can be put forward to explain the induction of Anx-A1 expression by E2 in MCF7 breast cancer cells – (1) Anx-A1 was up-regulated simply because other effectors upstream of Anx-A1 had been activated by E2 and that it plays no significant functional role in E2's effects on MCF7 cells; (2) E2 up-regulated Anx-A1 for pro-proliferative purposes and the resulting over-activation of certain signaling pathways (such as the MAPK pathway) caused anti-proliferative measures to be activated or (3) Anx-A1 is inherently, an anti-proliferative protein and its expression was increased to counter the over-activation of certain growth promoting signals caused by E2.

Our transfection studies have shown Anx-A1 to be intrinsically an anti-proliferative protein which may exert its effects in MCF7 cells via activating ERK constitutively. As can be construed thus, E2 could not have induced Anx-A1 expression for pro-proliferative purposes, as suggested by the second hypothesis. In addition, the results of the transfection studies are important for two other reasons. Firstly, they provide a causative role for Anx-A1 in the development of cancer. Since a basal level of Anx-A1 is required to put a brake on cellular proliferation (cells with Anx-A1 expression suppressed had increased proliferation rates) and that over-expression of Anx-A1 in

MCF7 cells led to decreased cellular proliferation, it would seem that at least in MCF7 cells, Anx-A1 has tumor suppressive functions. Secondly, they are in direct confirmation with Alldridge and Bryant's study which reported Anx-A1 to have a cell-type independent anti-proliferative function that acts through a sustained activation of the ERK signaling cascade, probably via an ERK-mediated disruption of the actin cytoskeleton and ablation of cyclin D1 protein expression (Alldridge and Bryant, 2003).

In addition, when MCF7 cells that had Anx-A1 expression suppressed (via transfection with Anx-A1 siRNA) were treated with E2, cells treated with 10^{-7} M and 10^{-9} M of E2 now showed the highest proliferation rates as compared to other cells - an observation which proved that the anti-proliferative role of Anx-A1 must have had been responsible, at least in part, for the reduced cell proliferation observed in cells exposed to high E2 doses over a prolonged period of time. This is hence, in direct opposition to the first hypothesis and supports the supposition that Anx-A1 was up-regulated to counter over-activation of pro-proliferative signals brought on by E2. Since Anx-A1 siRNA transfected cells were unable to sustain an activation of ERK after 72 hrs when treated with high E2 doses (unlike un-transfected and scrambled siRNA-transfected cells), Anx-A1 is apparently required for the constitutive ERK activation observed in cells treated with high E2 concentrations over a prolonged period of time. Removal of this constitutive ERK activation by suppressing Anx-A1 expression may hence eliminate the inhibition of cellular proliferation, and may thus explain why Anx-A1 siRNA-transfected cells treated with 10^{-7} M and 10^{-9} M of E2 now showed the highest proliferation rates as compared to other cells.

In summary, the experimental results generated within this study thus directly link Anx-A1 to a role in tumorigenesis (Figure 24). We have shown that prolonged exposure of MCF7 cells with E2 (after at least 48 hrs) can induce a dose-dependent up-regulation of Anx-A1, and that a higher expression of Anx-A1 induced by E2 was seemingly correlated with a lower rate of cell proliferation, despite the fact that E2 is a well-established growth stimulatory hormone in mammary cells. In addition, we have also shown that Anx-A1 has direct anti-proliferative effects on cells, possibly via a constitutive over-activation of ERK and that the anti-proliferative role of Anx-A1 is in turn, directly responsible, at least in part, for the reduced proliferation rates observed when cells were exposed to high E2 concentrations for a prolonged period of time. As such, we believe that Anx-A1 may act as a tumor suppressor in cells and that its expression may be increased in times of need to put a brake on cellular proliferation. Any mutations that occur in pathways that up-regulate Anx-A1 may hence render the cells incapable of inhibiting any uncontrolled proliferations and may thus in turn, explain why a decreased Anx-A1 expression has been implicated in breast cancer development and progression (Shen *et al.*, 2006).

As a side point, tamoxifen appears to act as an agonist within the context of our study as it not only failed to inhibit E2's induction of Anx-A1, but also stimulated proliferation of MCF7 cells instead of inhibiting it. As mentioned earlier, it is likely that tamoxifen has agonistic effects in this study since endogenous estrogens that compete with tamoxifen for ER-binding sites had been removed with the usage of charcoal-treated cell culture media. In line with this hypothesis, the addition of E2 to tamoxifen-treated

cells is likely to increase the number of competitors for ER-binding sites and may thus hence explain why cells treated with both E2 and tamoxifen typically demonstrated proliferation rates that were in between cells treated with either E2 or tamoxifen alone.

In addition, tamoxifen may stimulate proliferation of MCF7 cells via a pathway different from that of E2. The above hypothesis was made in lieu of the observation that treatment of the cells with tamoxifen had led to high cell proliferation rates despite a high level of ERK activation even after 72 hrs. In other words, while a prolonged ERK activation induced by high E2 concentrations may result in anti-proliferative signals being generated to curb uncontrolled cellular proliferation, a similarly high level of ERK activation in tamoxifen-treated cells could in contrast, possibly promote cell growth. However, since Anx-A1 has no apparent role to play in tamoxifen's effects on MCF7 cells, the mechanism of tamoxifen-induced cell proliferation was not examined in greater detail here.

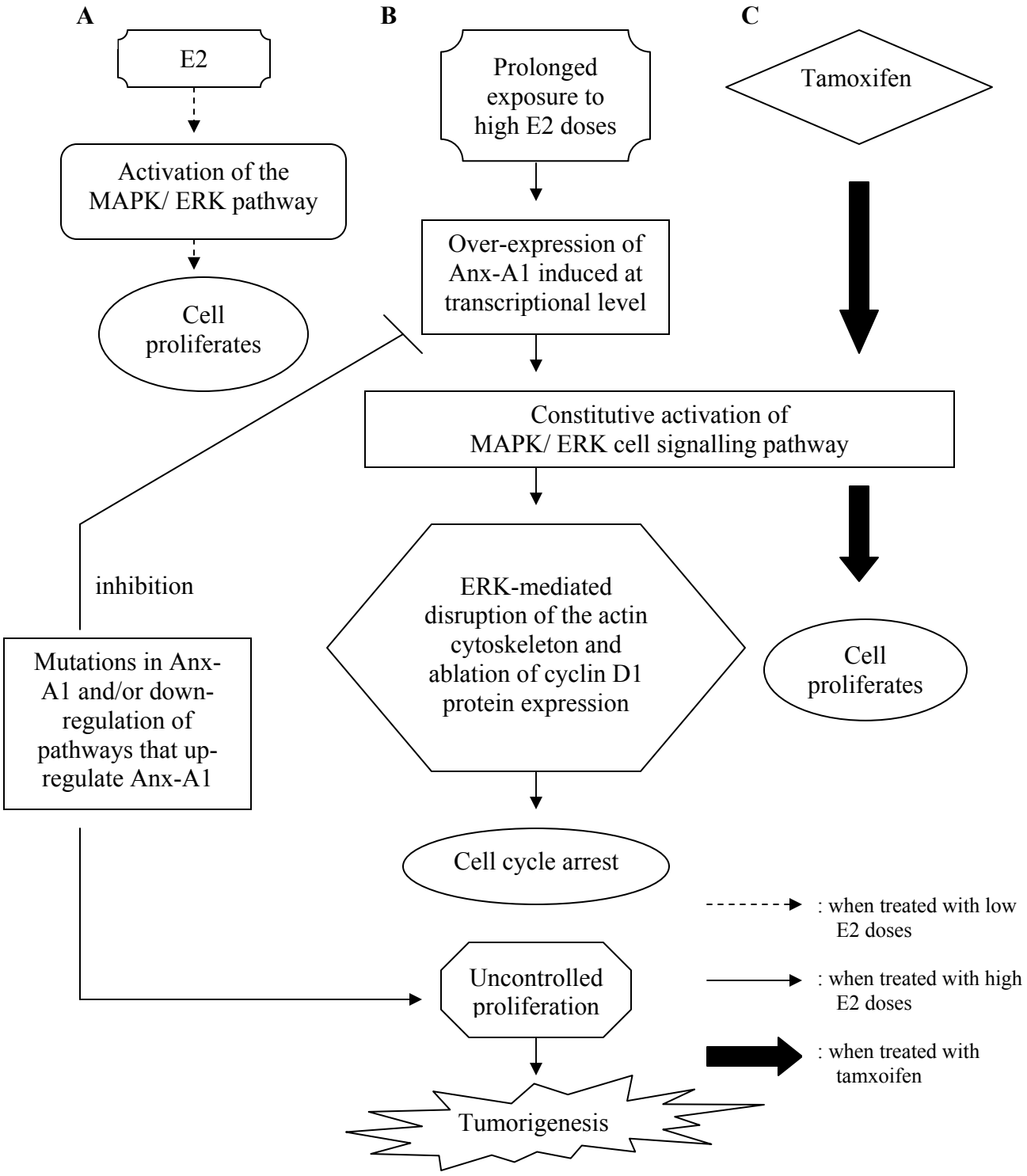


Figure 24: Simplified schematic diagram illustrating the possible role of Anx-A1 in breast tumorigenesis. (A): Possible scenario with low doses of E2; (B): Possible scenario upon over-expression of Anx-A1 induced by prolonged exposure to high E2 doses. A deregulation of pathways that increase Anx-A1 expression and/or loss-of-function mutations in Anx-A1 may result in uncontrolled cellular proliferation. (C): Possible scenario upon administration of tamoxifen.

Limitations of study and future work

A major limitation of this project is that the direct anti-proliferative signal responsible for the up-regulation of Anx-A1 has not been determined as at the conclusion of this study. As mentioned earlier, since the pro-proliferative effects of E2 on MCF7 cells have been well-documented by various studies, it is unlikely that E2 will increase the expression of Anx-A1 (which we had established as an anti-proliferative protein in MCF7 cells) for its purposes. Prolonged exposure to high E2 doses must have thus, generated anti-proliferative signals which induced the expression of Anx-A1. Yet, although high E2 doses led to a prolonged activation of ERK which may act as an anti-proliferative signal to up-regulate Anx-A1 expression, we have shown that this was not the case, since pre-treatment of the cells with U0126 inhibited ERK activation but not the induction of Anx-A1 by E2 after 72 hrs. The anti-proliferative signal to increase Anx-A1 expression remains to be elucidated and future work should concentrate on this aspect.

6. SUMMARY AND GENERAL CONCLUSIONS

- We have shown for the first time that E2 can induce Anx-A1 expression in MCF7 breast adenocarcinoma cells in a dose-dependent manner after at least 48 hrs.

- The induction of Anx-A1 is likely to be a result of gene transcription activated either directly or indirectly, by the binding of E2 to the estrogen receptor-alpha.

- A high expression level of Anx-A1 induced by E2 was correlated with a reduced rate of cell proliferation (which was not due to an increase in cell apoptosis mediated by Anx-A1). Relatively low levels (basal levels) of Anx-A1 in other drug-treated cells were however, correlated with an increased rate of cell proliferation.

- Treatment of cells with a high dosage of E2 led to an activation of ERK that was sustained even after 72 hrs and which could in turn act as an anti-proliferative signal. In contrast, cells treated with lower E2 doses had an activation of ERK which was not sustained after 72 hrs.

- Anx-A1 is required to sustain the ERK activation observed in cells treated with a high dose of E2 after 72 hrs.

- Anx-A1 has an inherent anti-proliferative role to play in MCF7 cells and that in line with observations previously reported by another group, Anx-A1 may possibly inhibit cell proliferation via a constitutive over-activation of ERK.

- The anti-proliferative role of Anx-A1 has a direct role to play in the reduced cell proliferation observed when cells were exposed to high E2 concentrations for a prolonged period.

- In conclusion henceforth, to the best of our knowledge, we have shown for the first time that:

  Treatment of MCF7 cells with high E2 doses over a prolonged period leads to the up-regulation of Anx-A1, which in turn, has direct anti-proliferative effects on MCF7 breast adenocarcinoma cells, possibly via a constitutive activation of ERK.

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