The Effects of the Progesterone Metabolites, 5α-pregnane-3,20-dione (5αP) and 3α-hydroxy-4-pregnen-20-one (3α-HP), on the Cytoskeleton and Cellular Adhesion of MCF-7 Breast Cancer Cells

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ABSTRACT

The steroid hormone, progesterone, is necessary for full differentiation and development of breast tissue. In both normal and cancerous breast tissue, progesterone can be metabolized into two separate categories of metabolites: the 5α-pregnanes, which stimulate cellular proliferation, and the Δ^4-pregenes, which inhibit cellular proliferation. Other than their effect on cellular proliferation, the effects of these progesterone metabolites on the structure and physiology of breast cancer cells are unknown. The objectives of this study were to determine the effects of 5α-pregnane-3,20-dione (5αP, a representitive 5α-pregnane) and 3α-hydroxy-4-pregnen-20-one (3α-HP, a representitive Δ^4-pregene) on cellular adhesion, actin cytoskeleton and focal adhesions, the expression of the structural proteins actin and vinculin, and the morphology of MCF-7 breast cancer cells.

The actin cytoskeleton and focal adhesions were studied in cells stained with Phalloidin-Rhodamine or anti-vinculin-TRITC using scanning laser confocal microscopy, flourescence microscopy, and digital computer image analysis. Unstained cells were utilized to study protein expression (via western blotting), cellular adhesion, and cellular morphology. Treatment with 5αP at concentrations as low as 10^{-8} M altered the cytoskeletal arrangement of MCF-7 breast cancer cells by decreasing the number of F-actin stress fibres and vinculin adhesion plaques by approximately 75%, decreasing expression of vinculin by approximately 75%, and by decreasing the polymerization of F-actin by approximately 80%. Cellular adhesion of breast cancer cells was also decreased by approximately 50%, and the cellular morphology of the cells was shifted from circular to a much more irregular shape. Treatment of MCF-7 breast cancer cells with 3α-HP at concentrations as low as $5X10^{-7}$ M was found to significantly increase cellular adhesion by approximately 40%, while displaying a trend towards increasing numbers of both adhesion plaques and stress fibres while cellular morphology, expression of vinculin, and expression of actin were not significantly altered.

These observations suggest that the progesterone metabolites 5αP and 3α-HP may play a role in breast cancer. 5αP induces alterations in the structure of MCF-7

cells, with decreased cellular adhesion and fewer cytoskeletal structures, alterations that resemble changes seen when normal breast cells become cancerous. Cells treated with 3α-HP induce alterations in MCF-7 breast cancer cells that are different from 5αP treated cells, with increases in adhesion and a slight increase in numbers of cytoskeletal structures. It is possible that these progesterone metabolites have different, and opposing, influences on the development of breast cancer.

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INTRODUCTION

1.1 Breast Cancer in Women

Breast cancer is the most common form of cancer in women, making up 29% of all cancer cases and causing 19% of cancer related deaths (Kelsey, 1993; Pasqualini and Katzenellenbogen, 1996). The occurrence of breast cancer on a global scale shows that women living in industrialized nations (North America, Europe, and Australia) exhibit an increased risk of developing the disease (Stevens and London, 1996). For a woman living in North America the chance of developing breast cancer is 1 in 9, with approximately 182,000 new cases being diagnosed in the United States in 1995 alone (Stevens and London, 1996). Despite modern therapies one-third to one-half of women who develop breast cancer will die from the disease (Berardo *et al.*, 1996).

Physiological, genomic, and endocrine factors contribute to the development and maintenance of breast cancer and breast cancer metastases (Pike *et al.*, 1993). In total over one hundred different prognostic factors for human breast cancer have been reported (Bonk, 1998). Physiological factors include age (Pike *at al.*, 1993), age at first full term pregnancy (Kelsey *et al.*, 1993), body build (Hunter and Willett, 1993), diet (Hunter and Willett, 1993), and family history of breast cancer (Kelsey, 1993). Genomic factors considered to be of importance are oncogenes (Walker *et al.*, 1997), growth factors (Ethier, 1995; Reid *et al.*, 1996), cell-extracellular matrix structures (Weaver *et al.*, 1997; German and Johanning, 1997), and plasminogen activators (Duggan *et al.*, 1995). Endocrine factors considered to be of importance include steroid hormones (Helzlsouer and Couzi, 1995; Lipworth *et al.*, 1995) and steroid hormone receptors (Donegan, 1992; Giangrande *et al.*, 1997). Of the steroid hormones the estrogens have attracted the bulk of the attention (Miller and Langdon, 1997), with progesterone and particularly progesterone metabolites receiving relatively little attention (Pasqualini and Katzenellenbogen, 1996; Wiebe *et al.*, 1999). If and what role progesterone metabolites play in the maintenance and development of breast cancer is not known at this time.

1.2 Steroid Hormones and Breast Cancer

Normal growth and development of breast tissue requires the steroid hormones estrogen (Leclerq and Heuson, 1979), and progesterone (Berardo *et al.*, 1996). Estrogens

have a strong stimulatory effect on cell proliferation in the breast, especially in ductal portions of the gland (Donegan and Spratt, 1995), while progesterone stimulation induces alveolar proliferation and cell growth in normal human breast tissue (Farrar, *et al.*, 1995). The action of progesterone on breast tissue is mediated and regulated by estrogens (Osborne, 1991) with the number of progesterone receptors and their affinity for binding progesterone being regulated by estrogen levels (Horwitz *et al.*, 1985).

The relationship between estrogens and breast cancer in women has been extensively studied while the relationship between progesterone and breast cancer is less clear (Berstein and Ross, 1993). Estrogens are able to increase the rate of proliferation in both *in vitro* and *in vivo* breast and breast cancer cells by inducing activity of growth factors (Walker *et al.*, 1997; DePasquale, 1994; Miller and Langdon, 1997). Estrogens have also been found to increase rates of breast cancer cell metastasis, the translocation of a cancer cell to a distant location within the body (Sapino *et al.*, 1986; DePasquale *et al.*, 1994).

There are conflicting reports about the role progesterone plays in the development and maintenance of breast cancer. Several studies involving progesterone have shown that when serum progesterone levels are at their highest, the rate of breast cancer proliferation is decreased and a regression of mammary gland tumors occurs (Osborne and Lippman, 1978; Leung *et al.*, 1980; King 1991). Other studies have shown that alveolar proliferation and cell growth is increased by low levels of progesterone (Farrar *et al.*, 1995; Soderqvist, 1998). *In vitro* studies have shown that progesterone has many different effects on the physiology of breast cancer cells including altering tissue structure (Gianelli *et al.*, 1999), inducing apotosis (Formby and Wiley, 1998), and affecting the expression of particular enzymes (Pasqualini *et al.*, 1998). One possible explanation for the varied effects of progesterone is that these effects may not be due to progesterone itself but to a combination of several different progesterone metabolites, each of which may have a different effect on the differentiation and growth of breast cells *in vitro*.

1.3 Metabolism of Progesterone Metabolites in Normal and Cancerous Breast Cells

Breast cells are able to metabolize and interconvert steroid hormones through their own enzyme systems (Wiebe *et al.*, 1999). Both normal and cancerous breast tissues are

able to convert pregnenolone to progesterone (Dao *et al.*, 1972), and androgens such as androstenedione and testosterone to estrogens (Miller *et al.*, 1974). The ability to interconvert steroids has also been noted in both non-tumorigenic and tumorigenic immortalized breast cell lines such as the MCF-7, T47-D, MDA-MB-231, and ZR-75-1 cells (Pasqualini *et al.*, 1989; Pasqualini *et al.*, 1992; Evans *et al.*, 1993).

Breast cells are able to covert progesterone into two different classes of steroid metabolites: 1) the Δ^4-pregnenes (which possess a $C_{4\text{-}5}$ double bond); and 2) 5α-pregnanes (which do not possess the double bond) (Lloyd, 1979; Hu, 1994; Wiebe *et al.*, 1999). Progesterone is converted irreversibly into 5α-pregnanes by the enzyme 5α-reductase (Wiebe *et al.*, 1999), while Δ-4-pregnenes are synthesized from progesterone via 3α-hydroxysteroid dehydrogenase and 20α-hydroxysteroid dehydrogenase enzyme activity (Wiebe *et al.*, 1999). Hormone synthesis differs greatly between tumorous and non–tumorous tissue, with the ratio of 5α-pregnanes/Δ^4-pregnenes progesterone metabolites being five fold greater in tumorous tissue (Wiebe *et al.*, 1999). Synthesis of 5α-pregnane-3,20-dione (5αP, a 5α-pregnane) and 3α-hydroxy-4-pregnen-20-one (3αHP, a Δ^4-pregnene), two of the primary products of hormone metabolism, also differs greatly between tumorous and non–tumorous tissue. Total endogenous 5αP, as determined by mass spectrometry, is greater in cancerous tissue (11.5 ng 5αP/mg tissue in cancerous breast tissue, 4.5 ng 5αP/mg tissue in normal breast tissue) while endogenous 3α-HP is greater in normal breast tissue (11.7 ng 3α-HP/mg tissue in cancerous breast tissue, 44.5 ng 3α-HP/mg tissue in normal breast tissue) (Hu, 1994). This difference in synthesis results in an approximate 24 fold increase in the ratio of 5αP/3α-HP in cancerous breast tissue in comparison to normal breast tissue (Wiebe *et al.*, 1999). The fact that progesterone metabolite synthesis is different between normal and cancerous tissues indicates that the differences could be due to the differences in concentration and metabolism of the progesterone metabolites.

1.4 Physiological Effects of the Progesterone Metabolites, 5αP and 3α-HP

Studies of the effects of several different 5α-pregnanes and Δ^4-pregnenes on MCF-7 and MCF-10A breast cancer cells have shown that 5α-pregnanes consistently stimulate

cell proliferation while Δ^4-pregnenes consistently inhibit cell proliferation in breast cancer cells (Wiebe *et al.*, 1999). 5αP has been found to increase proliferation 26%-110% at concentrations from 10^{-8}M to 10^{-6}M in the ZR-75-1 and MCF-7 breast cancer cell lines (Hu, 1994; Wiebe *et al.*, 1999). Little is known about the other effects 5αP might have on the physiology of the human body, although it has been suggested that progesterone competitively binds to progesterone receptors in breast tissue when in sufficient concentrations (Horwitz *et al.*, 1986). With its increased synthesis in cancerous breast tissue and its effect on cellular proliferation, 5αP could play an important role in the development and maintenance of breast cancer.

3α-HP has been found to inhibit cellular proliferation 20%-30% at concentrations from 10^{-8}M to 10^{-6}M in the ZR-75-1 and MCF-7 breast cancer cell lines (Hu, 1994; Wiebe *et al.*, 1999). In addition to its effect on breast tissue 3α-HP is known to stimulate spermatogenesis (Wiebe *et al*, 1988; Campbell and Wiebe, 1989), to selectively suppress FSH release from the pituitary (Wood and Wiebe, 1989; Dhavantari and Wiebe, 1994), and to act as a potent analgesic and anxiolytic (Wiebe and Kavaliers, 1988; Kavaliers, *et al.*,1994). With its proliferation inhibiting abilities 3α-HP could play a different role from 5α-P, possibly inhibiting the development of breast cancer.

The signal pathway through which 5αP and 3α-HP act has not been completely elucidated, though several important features have been determined. Membrane receptors exist for both 5αP and 3α-HP, with no significant hormone binding occurring with either cytosolic or nuclear steroid hormone receptors (Weiler, 1999). These receptors are specific to 5αP (K_d=4.5 nM) and 3α-HP (K_d=4.87 nm) with other progesterone metabolites, progesterone, and estrogen being unable to significantly displace 5αP or 3α-HP from their respective receptors (Weiler, 1999). In addition to membrane bound receptors 5αP and 3α-HP could also be acting through other steroid hormone receptor mechanisms such as the pregnane X receptor (PXR), a steroid hormone receptor that binds to pregnanes (Kliewer *et al.*, 1998). The pregnane X recepetor is able to bind to pregnanes such as progesterone and pregnenolone (Kliewer *et al.*, 1998) and could play a role in 5αP signaling in both normal and cancerous breast tissue.

To determine if progesterone metabolites are responsible for breast cancer or if differences in metabolite synthesis are due to some other factor the effects of 5αP and 3α-HP on MCF-7 breast cancer cells *in vitro* was tested (Wiebe *et al.*, 1999). If progesterone metabolites are responsible for alterations in cellular tumorigenicity, then alterations in cell structure similar to those in transformed cells should occur. Factors studied included cytoskeletal organization, cellular adhesion, and cellular morphology in order to determine if alterations to these characteristics matched those alterations that occur in cancerous cells.

1.5 Cellular Transformation: Effects on Cytoskeletal Organization, Cell Morphology, and Cellular Adhesion

Transformation of normal cells to cancerous cells, whether *in vitro* or *in vivo*, often results in dramatic changes in the organization of the cytoskeleton, morphology, and adhesive capabilities of the cell (Ben Ze'ev, 1985; Holth *et al.*, 1998). These changes can be seen in alterations in the organization of the actin cytoskeleton, distribution of cellular adhesion plaques, and resulting changes in cellular and nuclear morphology (Ben-Ze'ev, 1985; Holt *et al.*, 1998). Breast cancer cells, both *in vitro* and *in vivo*, display a much less organized cytoskeletal system and reduced cellular adhesion when exposed to estrogens, a steroid hormone which stimulates cell growth (Sapino *et al.*, 1985; Sapino *et al.*, 1986). Other than the effect on cellular proliferation, little is known about the effect of the lesser-known progesterone metabolites on breast cancer (Wiebe *et al.*, 1999). It is the purpose of this research to determine if the progesterone metabolites 5αP and 3α-HP have an effect on the organization of the actin cytoskeleton, adhesive capabilities, and morphology of breast cancer cells. If it is found that progesterone metabolites alter these structural elements in a way that is similar to the alterations that happen when a cell becomes cancerous it could indicate a link between progesterone metabolites and breast cancer.

1.5.1 Cellular Adhesion, the Cytoskeleton and Tumorigenicity

One of the key traits of transformed cells is the fact that many of them do not require cellular adhesion to grow, or if they require anchorage, it is to a much lesser extent than normal cells (Ben-Ze'ev, 1985; Holt *et al.*, 1998). This alteration of adhesion is a

necessary event for metastasis to occur, as the cell must be able to survive while detaching itself from the substrate and moving throughout the body (DePasquale *et al.*, 1994). Cellular adhesion is controlled by the cytoskeleton through dynamic structures termed adhesion plaques. Several different hormones such as melatonin (Matsui *et al.*, 1997), 1,25-dihydroxyvitamin D_3 (Segaert *et al.*, 1998), and estrogen (Sapino *et al.*, 1986) have been shown to decrease cellular adhesion in cells *in vitro* by altering the number and organization of the adhesion plaques.

Several types of intercellular adhesions maintain the integrity and organization of epithelial cell layers (DePasquale, 1994), including desmosomes, tight junctions, and intermediate junctions (DePasquale, 1994). The adherens junctions (intermediate junctions) consist of two generalized types of contacts: cell-to-cell and cell-to-matrix adherens junctions (DePasquale, 1994). The cytoplasmic side of both the cell-to-cell and cell-to-matrix adhesion plaques bind to actin filaments via several "linker" proteins (DePasquale, 1994). Both types of adhesion plaques contain several similar proteins such as vinculin (Geiger *et al.*, 1985), tenuin (Tsukita *et al.*, 1989), alpha-actinin (Lazarides and Burridge, 1975), along with several others (Depasquale, 1994).

Vinculin is a membrane-associated cytoskeletal protein localized at both the cell-to-cell and cell-to-substrate adhesive plaques (Sadano, 1992). This protein functions as a binding protein between the transmembrane proteins and actin fibers, although the physiological function and mode of interaction with molecules at the adhesion plaque such as integrin, talin, and α-actinin remains to be completely determined (Burridge, *et al.*, 1988; Sadano, *et al.*, 1992). Vinculin only occurs in polymerized form at the sites of cellular adhesion; unpolymerized vinculin is kept in equilibrium with the polymerized protein from a cytoplasmic pool (Geiger, 1985). By studying the localization of vinculin one can analyze the distribution and arrangement of adhesion plaques. One of the goals of this research is to determine if the cellular adhesion and organization of adhesion plaques in breast cancer cells *in vitro* are affected by treatment with the progesterone metabolites 5αP and 3α-HP.

1.5.2 The Actin Cytoskeleton and Tumorigenicity

In mammalian cells the actin cytoskeleton is responsible for many different cellular functions: cell motility and surface remodeling (Stossel, 1993), cell shape change during mitosis, muscle contraction and cytokinesis (Salmon, 1989), cell-cell and cell-substrate interactions with adhesion molecules (Luna and Hitt, 1992), endocytosis (Gottlieb *et al.*, 1993), and transmembrane signaling and secretion (Bretscher, 1993). Defects in any of these processes, whether due to disease or mutation, may be seen as changes in the cytoskeleton (Janmey and Chaponnier, 1995). Abnormal expression of actin and actin-bundling proteins has been implicated in cell transformation, alteration of metastatic potential, and cell motility (Mohandas and Evans, 1994; Janmey, 1998).

Actin cytoskeleton assembly is regulated at multiple levels, from monomers to polymers, and organization of polymers into a filamentous network. A large number of actin-binding proteins regulate actin assembly by controlling filament formation and crosslinking of the microfilaments (Dubreil, 1991). The actin associated proteins that crosslink actin filaments may function as regulators of cell growth and differentiation (Janmey and Chapponier, 1995).

In some types of cells transformation results in a decrease in the levels of actin associated proteins and also a change in cellular and nuclear morphology (Janmey *et al.*, 1995). The relationship of actin organization to transformation and metastasis varies between cell types. In T-lymphocytes the motility and F-actin content of non-invasive and invasive metastatic variants indicates that pseudopodia and filopodia formation requires a high levels of actin polymerization (Vershueren *et al.*, 1994). Pseudopodia and filopodia formation is vital if the cell is to invade other tissues, since cells unable to polymerize actin would be unable to metastasize. The opposite is true in rat sarcoma cells, where the cells displaying mostly short, fine actin filaments are the most motile and have the highest metastatic potential (Pokorna *et al.*, 1994). Excised cancerous breast tissue possesses a disrupted and disorganized F-actin cytoskeleton (Lin, 1993), although the metastatic capability of this tissue is not known. One of the goals of the present study is to determine if the actin cytoskeleton of breast cancer cells *in vitro* is affected by treatment with the progesterone metabolites 5αP and 3α-HP.

1.5.3 Cellular Transformation and Cytoplasmic Morphometry

The organization of the cytoskeleton is the driving force behind the cytoplasmic morphology of a particular cell, any alterations in cytoskeletal organization and arrangement can usually be seen as a change in cell morphology. Lewis (1939) attempted to describe cancer morphologically by reporting differences in the structural dynamics of cultured cells from normal and tumorous tissues. Due to the heterogeneity of the cultures he stated that thousands of cultures had to be observed before he could make the distinction between normal and tumor tissue (Giuliano, 1996). Early studies in morphometry revealed that transformation is accompanied by dramatic morphological changes (Fox *et al.*, 1977, Giuliano, 1996). Characteristics measured included were cell area, cell shape, migration, cell flattening and polarization, and presence of actin filament bundles (Folkman and Moscona, 1978; Forsby, *et al.*, 1985; Giuliano, 1996). Coupling morphological changes with the actual chemical events within the living cell with certainty had not been possible until recently (Giuliano, 1996). With the advent of computers it is now possible to describe critical cellular functions, both spatially and temporally, utilizing morphometry. A role for the actin-cytoskeleton in regulating normal cell functions such as cell division (DeBiasio *et al.*, 1996; Giuliano, 1996), locomotion (Post *et al.*, 1995; Giuliano, 1996), and signal transduction (Gough and Taylor, 1993, Giuliano, 1996) was recently determined utilizing morphometrical data. That changes in these critical cell functions can be visualized through alterations to their cellular and cytoskeletal morphologies suggests that a similar approach might be useful in studying and identifying the effects of progesterone metabolites on cancer cells *in vitro*. One of the goals of this research is to determine via morphometry if the cellular morphology of breast cancer cells *in vitro* is affected by the progesterone metabolites 5αP and 3α-HP.

1.5.4 Cellular Transformation and Nuclear Morphometry

The organization of the cytoskeleton is also one of the reasons that cells display a particular nuclear morphology. Alterations in nuclear morphology have long been a hallmark of cancer pathology (Pienta *et al.*, 1989), and are directly linked to the organization of the cytoskeleton. Researchers have sought an objective tumor evaluation

method of nuclear grading where certain features such as nuclear area and nuclear shape and cellular texture were measured (Becker, *et al.*, 1992). Problems with subjectivity and lack of a common sampling and classification system among pathologists led to other methods of classifying cell status. With the advent of Digital Computer Imaging Systems pathologists are now able to objectively study such factors as nuclear size and shape in fine needle aspirates of breast tissue (Wolberg *et al.*, 1997). Nuclear size, variation in nuclear size, and nuclear shape have been found to be strong prognostic indicators in the study and classification of breast cancer, even stronger than the previously used axial lymph node exam (Wolberg *et al.*, 1997). Prognosis of breast cancer is directly linked to the ability of the breast cancer cells to metastasize and form distant tumors (Wolberg *et al.*, 1997). One of the goals of this research is to determine via morphometry if the nuclear morphology of breast cancer cells *in vitro* is affected the progesterone metabolites 5αP and 3α-HP.

1.6 Cell Line Information

The MCF-7 cell line is a stable epithelioid cell line originally obtained from the pleural effusion of a female patient with metastatic breast cancer whose disease responded to hormone therapy (Soule *et al.*, 1973). The cells possess estrogen, progesterone, androgen and gluccocorticoid receptors and are frequently used as a model for estrogen dependent control of tumor cell function both in vitro and in vivo (Horwitz *et al.*, 1975). In most respects MCF-7 cells respond to steroid hormones in the same manner as cancerous breast tissue *in vivo*, resulting in the MCF-7 cell line being the cell line most commonly used in breast cancer research in the last 20 years (DePasquale, *et al.*, 1994). For this reason MCF-7 breast cancer cells were used to determine if the progesterone metabolites 5αP and 3α-HP have an effect on the organization of the actin cytoskeleton, cellular adhesion, and cellular and nuclear morphologies.

Hypothesis

The 5α-pregnane and Δ^4-pregnene progesterone metabolites are synthesized in differing amounts in normal and cancerous breast tissue, with cancerous tissue synthesizing dramatically more 5α-pregnanes relative to Δ^4-pregnenes. The 5α-

pregnanes (5αP) and Δ^4-pregnenes (3α-HP) stimulate and inhibit (respectively) the proliferation of breast cancer cells *in vitro*, and it is possible that they may play a role in the stimulation or inhibition of breast cancer. If the 5α-pregnane progesterone metabolites, such as 5αP, are a factor in causing breast cancer then alterations in the structure of breast cells treated with 5α-P should be similar to those that occur in cells that have become cancerous. If the Δ^4-pregnene progesterone metabolites, such as 3α-HP, are a factor in the inhibition of breast cancer then alterations in the structure of breast cells treated with 3α-HP should be different from the structure of cells that have become cancerous. The following study was undertaken to test the hypothesis that the progesterone metabolites 5αP and 3α-HP play a role the initiation and maintenance of breast cancer. If 5α-P and 3α-HP do play a role in breast cancer then cells treated with 5αP should and cells treated with 3α-HP should not display changes in cellular structure that are similar to changes that occur in cancerous cells. Changes that typically occur in cancerous cells include alterations in cell anchorage and adhesion, alterations in distribution and arrangement of the cytoskeleton, and alterations in cytoplasmic and nuclear morphology.

The specific objectives of this study were:

1) To determine the effect of progesterone metabolites, 5α-P and 3α-HP, on the cellular adhesion of MCF-7 breast cancer cells.

2) To determine the effect of progesterone metabolites, 5α-P and 3α-HP, on the architecture of the actin cytoskeleton and vinculin containing adhesion plaques in MCF-7 breast cancer cells.

3) To determine the effect of progesterone metabolites, 5α-P and 3α-HP, on the cellular expression of actin and vinculin within MCF-7 breast cancer cells.

4) To determine the effect of progesterone metabolites, 5α-P and 3α-HP, on the cytoplasmic and nuclear morphology of MCF-7 breast cancer cells.

MATERIALS AND METHODS

2.1 Cell Culture

2.1.1 Chemicals and Materials

All chemicals, materials and their sources are listed in Appendix 1. 5αP was purchased from Sigma Chemicals (St. Louis, MO) while 3α-HP was synthesized in this laboratory (Wiebe *et al.*, 1985).

2.1.2 Cell Culture Conditions

MCF-7 human breast adenocarcinoma cells were obtained from the Michigan Cancer Foundation (Detroit, Michigan) at passage 134. The cultures were maintained in 75 cm^2 flasks in Dulbecco's Minimal Essential Medium (DMEM): F12-Ham containing 5% bovine calf serum, 0.25μM $CaCl_2$, 0.006% penicillin, 0.01% streptomycin, 0.001% insulin, 0.11% Fungizone, 14.3 mM sodium bicarbonate, pH 7.2. Cultures were maintained at 37°C with 100% humidity in a humidified incubator within a 5% CO_2 atmosphere.

Cells were harvested at approximately 80% confluence using 0.1% Trypsin and 0.05% EDTA in phosphate buffered saline (PBS; pH 7.2). Only cells between passage 136 and 146 were used for morphology, protein expression, and cellular adhesion experiments.

2.1.3 Experimental Conditions

Seventy-two hours before starting any experiment cells were transferred to media containing BCS stripped of endogenous hormones (experimental media). BCS was stripped by stirring at 4°C for twelve hours with 1% activated charcoal and 0.1% Dextran T-70. BCS was then centrifuged at 10,000 x g for 15 minutes, decanted, and filtered through a sterile 0.2μm syringe filter.

2.2 The Effect of 5αP and 3α-HP on Cellular Adhesion

2.2.1 Detachment

After 72 hours in experimental media cells were transferred to 35mm plastic petri dishes (Falcon), with approximately 10,000 cells/dish. Cells were grown in experimental media with the appropriate concentration of 5αP, 3αHP, or without added hormones

(control). Cells were then cultured for 72 hours, with the media being replaced every 24 hours in order to replace metabolized hormones.

After 72 hours cultures were removed from the humidified incubator and rinsed twice with 37°C PBS. Cultures were then incubated with 1.0ml of 0.1%Trysin with 0.5% EDTA for 6 minutes at room temperature, after which 1.0 ml of experimental media was added to neutralize the Trypsin/EDTA. Dishes were then placed on a rotating shaker at 60 rpms for 1 minute, from which the media/Trypsin solution was removed using a Pasteur pipette and placed in a test tube. Cells collected in this sample were considered to have detached from the substrate due to the Trypsin/EDTA treatment. Another 1.0ml of Trypsin/EDTA was added to the dish and the culture was incubated for 4 minutes at rt, after which 1.0ml of media was added to the culture to neutralize the Trypsin/EDTA. Using a Pasteur pipette to gently wash the remaining cells off the plastic dish the media/Trypsin solution was collected and placed in a test tube. Cells collected in this sample were considered to have not detached from the substrate due to Trypsin/EDTA treatment. Samples were then centrifuged at 50 x g for 10 minutes to collect the cells in the bottom of the tube, and the Trypsin/EDTA solution was decanted. The cells were then resuspended in 500 µl of media:stripped BCS and counted using a Neubauer haemocytometer. Data was expressed as percentage of total cells that had detached from the plastic substrate.

2.4.2 Attachment

After 72 hours of growth in experimental media cells were subcultured to 75cm^2 plastic culture flasks (Falcon), with approximately 3 X 10^6 cells/flask. Cells were cultured for 72 hours in media containing either no additional hormones (control), 5αP, or 3αHP with the media being changed every three days to replace metabolized hormones.

After 72 hours the culture flasks were removed from the humidified incubator and the cells subcultured into 35 mm plastic petri dishes in media:stripped BCS with no additional hormones (approximately 10,000 cells/dish). The dishes were then incubated at room temperature to allow the cells to attach to the plastic substrate. After 2.5 hours the cultures were placed on a rotating shaker at 60 rpms for 1 minute, after which the media was collected with a Pasteur pipette and placed in a test tube. Cells in this sample were considered not to have attached to the substrate. Cells remaining in the culture dishes were

then collected using Trypsin/EDTA and a Pasteur pipette and placed in a test tube (see 2.8.1). Cells in this sample were considered to have attached to the substrate. Samples were then centrifuged at 50 x g for 10 minutes to collect cells at the bottom of the tube and the solution was decanted. The cells were resuspended in 500µl of media:stripped serum and counted with a Neubauer haemocytometer. Data was expressed as percentage of total cells in dish that had attached from the substrate.

2.3 Histochemical Staining for Adhesion Plaques and Actin Stress Fibers

2.3.1 Experimental Conditions

After 72 hours of culture in experimental media cells were subcultured into 35 mm plastic petri dishes containing 22 mm X 22 mm glass coverslips (approximately 10,000 cells/dish). Cells were cultured for 72 hours in experimental media containing 5αP, 3αHP, or no additional hormones (control). Culture media were changed every 24 hours to replace metabolized hormones.

2.3.2 Staining for Actin Stress Fibers

The protocol for staining for filamentous actin was taken from Clubb (1993). In short, 72 hour old cultures were rinsed for ten minutes in two changes of 37°C PBS before being fixed in a 37°C 2% formaldehyde:PBS for fifteen minutes. Cells were then rinsed in two 10 minute washes in PBS at room temperature and then treated for 5 minutes with 0.1% Triton X-100 (Sigma). Cultures were then rinsed in three 5-minute washes of rt PBS.

Phalloidin-TRITC was diluted 1:20 in PBS, resulting in a working concentration of 0.05 mg/ml. Coverslips were taken from the dishes, blotted dry, and placed cell side down over a 50 µl drop of the Phalloidin-TRITC solution in a sterile dish and incubated for 25 minutes at 4°C in a humidity chamber. The stained cells were then rinsed in three 5-minute washes of rt PBS and mounted onto glass slides using glycerol:PBS (9:1, pH 9).

2.3.3 Staining for Vinculin Adhesion Plaques

Seventy-two hour old cultures were rinsed twice in PBS (37°C) before being fixed in formaldehyde (2% in PBS; 37°C) for seven minutes. Cells were then rinsed for two 5-minute washes with rt PBS before being treated with 0.1% Triton X-100 for 7 minutes.

Cultures were then rinsed for three 5-minute washes with PBS and blocked for 15 minutes with 1% Bovine Serum Albumin (BSA) in PBS (blocking buffer).

A mouse monoclonal anti-vinculin antibody was diluted 1:50 in blocking buffer and was used as the primary antibody solution. Coverslips were taken from the dishes, blotted dry, and placed cell side down over a 50 µl drop of the primary antibody in a sterile dish. Cultures were then incubated for 1 hour in a room temperature humidity chamber, after which they were rinsed with three 5-minute washes of room temperature blocking buffer. Coverslips were then placed cell side down over a 50 µl drop of the secondary goat anti-mouse IgG-antibody conjugated to Phalloidin-TRITC diluted 1:25 with PBS. The cultures were then incubated for 1 hour in a room temperature humidity chamber, rinsed with three 5-minute washes of room temperature PBS before being mounted on glass slides with glycerol:PBS (9:1).

2.3.4 Sampling and Photomicroscopy of Stress Fibers and Adhesion Plaques

Observations were made of individual cells, subconfluent cultures, and confluent cultures of MCF-7 cells. These three separate categories were selected to show the differences that cell crowding cause in the cytoskeletal organization of MCF-7 cells. Characteristics of actin filaments that were observed included: filament presence and number, filament organization, and complexity of the filament network. Anti-vinculin stained cells were characterized according to the presence, location, and number of vinculin containing adhesion plaques. Cells were examined with a Biorad MRC-600 Laser Scanning Confocal Microscope. Observations were made using both a 60x objective and 40x objective lens, while photomicrographs were taken using the 40x objective lens.

2.4 Quantification of Numbers of Stress Fibers and Adhesion Plaques Via Flourescent Microscopy

2.4.1 Counting of F-actin Stress Fibers

Selected cells were magnified until they filled the field of view. Stress fibers were then counted in each cell, with attempts being made to find both ends of the filament. Filaments were counted if they were a) linear, b) well defined within the cell, and c) if both ends of the filament were visible.

2.4.2 Counting of Adhesion Plaques

Selected cells were magnified until they filled the field of view and any adhesion plaques within the cell or at the perimeter of the cell were counted.

2.4.3 Sampling of Stress Fibres and Adhesion Plaques

At a 40X magnification 3 fields of view were chosen randomly from the slide. Within each field five cells were chosen, the one closest to the middle of the field, the others closest to the points corresponding to the compass points NE, NW, SE, SW.

2.5 Quantification of Actin Stress Fibres and Vinculin Adhesion Plaques Via Quantitative Fluorescence Image Analysis

2.5.1 Quantitation of Fluorescence via Image Analysis

Fluorescence brightness of stress fibers and adhesion plaques in Rhodamine stained cells was calculated using the DCIS Northern Exposure. The DCIS program was calibrated to the 40x objective lens to determine both the total number of pixels/cell and the optical grey scale of each individual pixel. The average optical grey scale /pixel was calculated for each cell scanned, this grey scale/pixel value represents the average brightness of fluorescence in each cell. Greater fluorescence indicates greater amounts of polymerized actin or vinculin within the cell.

2.5.2 Control for Background

In order to eliminate background staining from the fluorescence value, one coverslip per treatment group was used as a background control. One coverslip of each treatment group that was to be studied for stress fibers was treated with 10^{-6} M Cytochalasin D for 10 minutes before staining with Phalloidin-Rhodamine. Treatment with Cytochalsin D depolymerized any stress fibres so that only background staining occurred within the cell. One coverslip of each treatment group that was to be studied for vinculin adehsion plaques was treated with only goat anti-mouse IgG secondary antibody. Not treating the cells with primary anti-vinculin antibody resulted in only background staining occurring within the cell. An average fluorescence value was determined for the background coverslip for each

treatment group and was subtracted from the average treatment group fluorescence to determine fluorescence due to specific actin and vinculin staining.

2.5.3 Sampling for Quantitative Fluorescence Image Analysis

Using a 40X objective lens, 10 fields of view were chosen in a 2 X 5 grid on the microscope slide. Within each field five cells were chosen, the one closest to the middle of the field, the others closest to the points corresponding to the compass points NE, NW, SE, SW.

2.6 The Effect of 5αP and 3α-H on Protein Expression of Actin and Vinculin in MCF-7 Cells

2.6.1 Experimental Conditions

After 72 hours of culture in experimental media, approximately 3 X 10^{-6} cells were transferred to a T-75 plastic culture flask. Cells were cultured for 72 hours, to approximately 80% confluence, in experimental media containing no additional hormones (control), 5αP, or 3αHP. Culture media were changed every 24 hours to replace metabolized hormones.

2.6.2 Protein Determination

Protein concentration of samples was determined in order to load each gel lane with equal amounts of cellular protein. Briefly, cells were collected from an 80% confluent T-75 culture flask that had been grown in parallel with the treatment flasks. Cells were collected from this flask via scraping with a rubber policeman, boiled for 10 minutes in an Eppendorf tube and passed through the end of a 22 gauge needle. Protein concentration was then determined via the method of Bradford (Butler & Dawson, 1992).

2.6.3 Sample Preparation for Actin and Vinculin Expression

Culture media were removed and 500μl of Lysis Buffer was added to a single T-75 culture flask of 80% confluent MCF-7 cells. Lysis Buffer consisted of 50 mM Tris-HCl, 100 mM Dithiothrietol, 2% Sodium Dodecyl Sulphate (SDS), 10% glycerol , 1 mM PMSF and 0.1% bromophenol blue. Cell lysate was collected and boiled for 10 minutes in a

plastic 1.5 ml microcentrifuge tube, after which it was repeatedly passed through a 21 gauge needle on ice to shear any gelatinous DNA. Care was taken not to introduce air bubbles to the lysate. The lysate was then centrifuged at 10,000 x g for 10 minutes after which the supernatant was decanted, and then loaded into the SDS-polyacrylamide gel.

2.6.4 Isolation of Insoluble Actin

Approximately 15 million cells were lysed with 3.0 ml of stock Triton solution (2% Triton X-100, 160 mM KCl, 40 mM imidazole-HCL, 20 mM EGTA, 8 mM sodium azide, pH 7.0) and collected into 1.5 ml eppendorf tubes. Tubes were placed on ice for 10 minutes and then centrifuged for 10 minutes at 10,000g, after which the supernatant was decanted and saved for isolation of soluble actin (section 2.7.2.3). The pellets were then dissolved in 250µl aliquots of loading buffer (2% SDS, 100mM dithiothrietol, 50 mM Tris-HCL, 10% glycerol, 1 mM PMSF, 0.1% bromophenol blue, pH 6.8), incubated in a boiling water bath for 10 minutes, and vortexed vigorously for 1 minute. The resulting solution contained the insoluble actin (F-actin) from within the MCF-7 cells.

2.6.5 Isolation of Soluble Actin

One ml aliquots of supernatant from isolation of insoluble actin were added to 300 µl aliquots of running buffer and boiled for 5 minutes. The resulting solution contained the soluble actin (G-actin) from within the MCF-7 cells.

2.6.6 Polyacrylamide Gel Electrophoresis

The cellular proteins were electrophoresed through a 12% polyacrylamide mini-gel system (Biorad) according to the protocol of Thomas and Kierney (1975).

2.6.7 Western Blotting for Actin and Vinculin

Cytoskeletal proteins were transferred to a nitrocellulose membrane (Biorad) overnight at 4°C according to the protocol of Thomas and Kierney (1975). After transfer the nitrocellulose membranes were blocked for 1 hour in blocking buffer (1% bovine serum albumin (BSA) in PBS. Nitrocellulose blots were then incubated with either primary anti-actin antibody at a 1:100 dilution or primary anti-vinculin antibody at a 1:40

dilution in blocking buffer for 2 hours at 4°C with gentle agitation. After incubation, the nitrocellulose blots were washed with three ten minute rinses of blocking buffer and transferred to a solution of secondary goat anti-mouse IgG conjugated to alkaline phosphatase at a dilution of 1:2000 in blocking buffer.

2.6.8 Western Blotting for Ornithine Decarboxylase

Western Blotting for ornithine decarboxylase proceeded as with actin and vinculin, with the following exceptions. Nitrocellulose membranes were incubated with anti-ornithine decarboxylase antibody at a 1:80 dilution in blocking buffer. The control Western blot for ornithine decarboxylase expression was transferred from a different SDS-PAGE gel loaded with the same amount of sample that the actin expression and organization Western Blot was loaded with. The actin and ornithine decarboxylase bands, if developed on the same blot, would be too close together to separate. The ornithine decarboxylase Western Blot control for vinculin expression was taken from the same SDS-PAGE as the vinculin expression western blot.

2.6.9 Western Blotting Alkaline Phosphatase Detection System

All Western blots were developed with the goat anti-mouse Alkaline Phosphatase (AP) Western blot visual detection kit.

2.6.10 Quantitation of Western Blot Banding via Image Analysis

All western blots were scanned into the image analysis and manipulation software package Photoshop. Grey scale optical densities of the center of each band (vinculin, actin, or ornithine decarboxylase) were then calculated.

2.7 Effects of 5αP and 3α-HP on Cell Morphology

2.7.1 Experimental Conditions

After 72 hours of culture cells were seeded onto acid washed (10% HCL in 90% Ethanol) 22 X 22 mm glass coverslips in 35 mm plastic petri dishes (approximately 10,000 cells/dish). Cells were cultured for 72 hours in experimental media containing the appropriate concentration of 5α-pregnane-3,20-dione (5αP), 3α-hydroxy-4-

pregnen-20-one (3αHP), or no additional hormones (control). Culture media were changed every 24 hours to replace metabolized hormones

2.7.2 Cytoplasmic Staining

For the study of cell and nuclear morphology, MCF-7 cells were stained with Ehrlich's Haemotoxylin and Eosin.

2.7.3 Sampling and Microscopy

Observations were made of individual, subconfluent, and confluent cultures of MCF-7 cells. Cells in three different degrees of cell crowding were studied in order to determine if cell crowding affected cell morphology. Morphological characteristics observed were (1) overall shape of cells, (2) presence/absence of peripheral cell structures such filopodia and lameleipodia, and (3) interaction of cells with neighbouring cells . Cells were studied using a Leitz DMRBE Light Microscope (Leica).

2. 8 The Effects of 5αP and 3α-HP on Cytoplasmic and Nuclear Morphology

2.8.1 Cytoplasmic Staining

Both cell and nuclear area and shape, being the most commonly used morphometric features, were studied in breast cancer cells treated with 5αP and 3α-HP. For the study of cell area and cell shape using the Digital Computer Imaging System (DCIS) Northern Exposure (copyright 1995, Imagexperts), MCF-7 cells were fixed in 2% formaldehyde:PBS (pH 7.2) for 5 minutes and then stained with Giemsa stain (2% stock Giemsa and 4% stock buffer, pH 6.5). For study of nuclear area and nuclear shape MCF-7 cells were stained with Ehrlich's Haemotoxylin.

2.8.2 Cell Area

Cell area was calculated using the DCIS Northen Exposure. The DCIS program was calibrated to the 40x objective lens and the area/screen pixel was calculated. The DCIS then determined the total number of screen pixels contained within the image of each MCF-7 cell selected and the total cell area was calculated.

2.8.3 Cell Shape

Index of cellular shape was calculated using the DCIS Northern Exposure. The DCIS program was calibrated to the 40x objective lens and the area/screen pixel was calculated. The DCIS then determined both the total number of screen pixels within the scanned MCF-7 cell and the total number of pixels making up the perimeter of each MCF-7 image. Total cell area, cell perimeter and cell shape (Equation 1) were then calculated.

$$\text{Equation 1: Cell shape} = \frac{(\text{Perimeter})^2}{4 \times \text{Pi} \times \text{Area}}$$

Note: This equation gives a relative value of the shape of a cell based on the ratio of cell perimeter to cell area. Cell shape for a perfect circle is 1.000 (minimum perimeter/maximum area) while the index of cellular shape for a straight line would approach infinity (perimeter increasing as the area decreased). As shapes become more complex (increase perimeter relative to area) the cell shape will increase.

2.8.4 Nuclear Area

Nuclear area was determined in the same manner using the DCIS Northern Exposure as was peformed for determining cell area (see section **2.8.2**).

2.8.5 Nuclear Shape

Nuclear shape was determined in the same manner using the DCIS Northern Exposure as was performed for determining cell shape (section **2.8.3**).

2.8.6 Sampling and Microscopy

Observations were made of single MCF-7 cells which did not come into contact with neighboring cells. This was done to ensure that the area and morphological features of the cell were due to the cytoskeletal architecture of the cell itself and not due to crowding by other cells. All observations were made using the 40x objective lens of an Orthoplan

Photomicroscope equipped with epiflourescence (Leitz, Germany).

2.9 Cell Mortality

After 72 hours of growth in experimental media cells were subcultured to 75cm^2 plastic culture flasks (Falcon), with approximately 3 X 10^6 cells/flask. Cells were cultured for 72 hours in media containing either no additional hormones (control), 5αP, or 3αHP with the media being changed every three days to replace metabolized hormones.

Cells were then removed from the growth flask (using Trypisn/EDTA) and counted on a Neubauer Haemocytometer to determine cell mortality due to steroid hormone treatment via the Trypan Blue exclusion protocol for living cells (Butler and Dawson, 1992).

RESULTS

3.1 Effect of 5αP and 3α-HP on Cellular Adhesion

Both 5αP and 3α-HP dramatically affected the ability of MCF-7 breast cancer cells to adhere to the substrate. Both of these hormones were able to alter cellular adhesion in a dose dependent manner, with 5αP decreasing and 3α-HP increasing cellular adhesion.

3.1.1 Detachment

Treatment of MCF-7 cells with 5αP at a sufficient concentration significantly altered the ability of cells to detach from the plastic growth substrate. Concentrations of 5αP between 10^{-10}M and 10^{-8}M had no effect on the ability of the cells to detach from the substrate (Fig.1). Concentrations between 10^{-8} M and 10^{-7} M resulted in a dose dependant increase in the rate of detachment of the cells from the substrate (Fig 1.). Approximately 65% of cells detached from the substrate after treatment with 5αP in concentrations between 2 X 10^{-7}M and 10^{-6}M, an increase of 20% relative to control cells (Fig. 1). Concentrations of 5αP from 10^{-6} M to 10^{-4} M also resulted in a 20% increase in rate of detachment from the substrate (results not shown).

Concentrations of 3α-HP between 10^{-10}M and 10^{-8}M had no effect on cell detachment while treatment with concentrations of 3α-HP between 10^{-8} M and 5X10^{-7} M resulted in a dose dependent decrease in the detachment of the cells from the substrate (Fig 1.). Approximately 33% of cells detached from the substrate after treatment with 3α-HP in concentrations between 5 X 10^{-7}M and 10^{-6}M, a decrease of approximately 15% relative to control cells (Fig. 1). Concentrations of 3αHP greater than 10^{-6}M results in an increase in rate of cellular detachment of approximately 15%, though concentrations higher than 10^{-5} M show an increase in rate of cellular detachment (results not shown) that coincides with increased levels of cellular mortality (Appendix 2).

3.1.2 Attachment

Treatment of MCF-7 cells with 5αP at high enough concentrations significantly altered the ability of cells to attach to the plastic growth substrate. Treatment of cells

with concentrations of 5αP between 10^{-10}M and 8X10^{-7}M did not affect the ability of the cells to attach to the substrate (Fig. 2). Treatment of breast cancer cells with 5αP between 7X10^{-7} M and 8X10^{-6} M resulted in a dose dependent decrease on the rate of attachment of the cells to the substrate (Fig 2.). Approximately 35% of cells attached to the substrate after treatment with 5αP in concentrations between 8X10^{-6}M and 10^{-6}M, a decrease of 20% relative to control cells (Fig. 2). Concentrations of 5αP from 10^{-6} M to 10^{-4} M also resulted in a 20% decrease in rate of attachment to the substrate (results not shown).

Concentrations of 3α-HP between 10^{-10}M and 6X10^{-7}M had no effect on the ability of the cells to attach to the substrate. Treatment of breast cancer cells with 3α-HP at concentrations between 7X10^{-7} M and 5X10^{-6} M resulted in a dose dependent increase in the attachment of the cells to the substrate (Fig 2.). Approximately 70% of cells attached to the substrate after treatment with 3α-HP in concentrations between 5 X 10^{-7}M and 10^{-6}M, an increase of approximately 10% relative to control cells (Fig. 2). Concentrations of 3αHP greater than 10^{-6}M resulted in an increase in rate of cellular detachment of approximately 10%, though concentrations higher than 10^{-5} M show a decrease in rate of cellular attachment (results not shown) that coincides with increased levels of cellular mortality (Appendix 2).

3.2 The Effect of 5αP and 3α-HP on Adhesion Plaques

Both numbers of adhesion plaques (quantitated through counts and digital computer image analysis) and adhesion plaque arrangement were significantly altered in a dose dependent manner. Treatment of MCF-7 cells with sufficient concentrations of 5α-P dramatically reduced the number and size of adhesion plaques. Cells treated with 3α-HP did not show a significant alteration in numbers or arrangement of adhesion plaques, though there was a slight trend towards increasing numbers of adhesion plaques at higher concentrations.

3.2.1 Adhesion Plaque Arrangement

Untreated MCF-7 cell focal adhesions occur in a regular pattern, the arrangement of which depends on the degree of cellular crowding. Several different degrees of

cell crowding were studied, including lone cells, subconfluent cultures, and confluent cultures to determine if there were differences in adhesion plaque arrangement. Alterations in adhesion plaque distribution did occur with cell crowding. Lone, control MCF-7 cells arranging adhesion plaques as small dash or dot shaped plaques, primarily at the periphery of the cell, with little basal layer adhesion (Fig 3A and 3B). No differences were seen in adhesion plaque arrangement between subconfluent and confluent cultures. Subconfluent and confluent MCF-7 cells arranged adhesion plaques as small plaques both peripherally and basally (Fig 4A and 4B), with plaques possessing a more dash shaped morphology with few dot shaped adhesion plaques.

Treatment of lone and subconfluent cells with 10^{-6} M 5α-P resulted in an almost complete loss of adhesion plaques relative to control cells (Fig. 3D and 4D), while treatment of similar cultures with 10^{-10} M 5α-P showed no difference from control cells (Fig. 3C and 4C). Increasing concentrations of 5α-P (10^{-8}M to 10^{-5}M) resulted in a dose dependant trend towards decreased arrangement of adhesion plaques, with fewer adhesion plaques present within the cell (results not shown).

Treatment of lone and subconfluent cells with 10^{-6} M 3α-HP resulted in no observable changes in arrangement of adhesion plaques relative to control cells (Fig. 3F and 4F), while treatment of similar cultures with 10^{-10} M 3α-HP also showed no difference from control cells (Fig. 3E and 4E). Concentrations of 3α-HP between 10^{-10} M and 10^{-5}M also did not display any difference in adhesion plaque arrangement between treated and control cells (results not shown).

3.2.2 Numbers of Adhesion Plaques

Analysis of cell adhesion plaque numbers revealed that MCF-7 cells treated with 10^{-6} M 5α-P had significantly fewer (approximately 75%) adhesion plaques than either control cells, cells treated with 10^{-10} M 5α-P, or cells treated with 3α-HP ($p<0.01$, Fig. 5). Further analysis has shown that concentrations of 5α-P from 10^{-8}M to 10^{-6}M cause a significant dose dependent decrease in numbers of adhesion plaques (Fig. 6). Concentrations of 5αP from 10^{-6}M to 10^{-5}M caused a constant 75% decrease in numbers of adhesion plaques (Fig. 6).

Treatment with 3α-HP at concentrations of 10^{-10} M and 10^{-6} did not cause a significant alteration in adhesion plaque numbers relative to control cells (Fig.5). Treatment of cells with concentrations of 3α-HP between 10^{-7}M and 10^{-5}M show a slight trend towards an increase in numbers of adhesion plaques (Fig. 6), though the trend is not seen in at lower concentrations between 10^{-10}M and 10^{-8}M.

3.2.3 Quantitiation of Adhesion Plaque Fluorescence via Image Analysis

Quantitation of fluorescence in Rhodamine stained cells is a method to quantify the relative amounts of vinculin containing adhesion plaques between different treatment groups. Digital computer image analysis of cell adhesion plaque fluorescence revealed that MCF-7 cells treated with 10^{-6} M 5α-P had significantly lower (approximately 50%) adhesion plaque fluorescence than either control cells, cells treated with 10^{-10} M 5α-P, or cells treated with 3α-HP ($p<0.01$, Fig. 7). Further analysis has shown that concentrations of 5α-P from 10^{-8}M to 10^{-7}M cause a significant dose dependant decrease in fluorescence of adhesion plaques (Fig. 8). Concentrations of 5αP from 10^{-6}M to 10^{-5}M caused a consistent 60% decrease in adhesion plaques fluorescence (Fig. 8).

Treatment with 3α-HP at concentrations of 10^{-10} M and 10^{-6} did not cause a significant alteration in adhesion plaque fluorescence relative to control cells (Fig.7). Treatment of cells with concentrations of 3α-HP between 10^{-10}M and 10^{-6}M show a slight trend towards an increase in fluorescence of adhesion plaques, though the trend is not seen at 10^{-5}M (Fig. 8).

3.3 Effects of 5αP and 3α-HP on Stress Fibres

Both numbers of stress fibres and stress fibre arrangement were significantly altered in a dose dependant manner. Treatment of MCF-7 cells with sufficient concentrations of 5α-P dramatically reduced the number stress fibres. Cells treated with 3α-HP did not show a significant alteration in numbers of stress fibres or arrangement of stress fibres, though there was a slight trend towards increasing numbers of stress fibres some of the higher concentrations.

3.3.1 Stress Fibre Arrangement

Stress fibre arrangement within MCF-7 cells is strongly dependent upon degree of cellular crowding. Several different degrees of cell crowding were studied, including lone cells, subconfluent cultures, and confluent cultures to determine if there were differences in stress fibre arrangement. The most common form of cytoskeletal organization in untreated, lone cells was a peripheral ring of actin stress fibers occurring tangentially to each other (Fig 9A and 9B), with few fibres crossing the body of the cell. Stress fibres in a peripheral ring arrangement were almost exclusively bound to locations at the periphery of the cell, with very few fibres binding to the basal layer. Occurring less frequently was the trans-cellular fiber system, which consists of parallel fibers crossing the body of the cell (pictures not shown) and binding to both the cell periphery and the basal cell membrane. Subconfluent cultures of MCF-7 cells organized actin stress fibers in both peripheral ring and trans-cellular patterns, with the majority of peripheral ring structures occurring within cells at the edge of cellular clusters (Fig. 10A and 10B). The majority of cells in confluent cultures of MCF-7 cells organized stress fibers in a trans-cellular pattern (Fig. 11A and 11B).

Lone, subconfluent, and confluent cells treated with 10^{-6} M 5α-P showed an almost complete loss of observable stress fibers (Fig. 9D, 10D, 11D) while treatment with 10^{-10} M 5α-P showed no apparent difference from control cells in regards to stress fiber arrangement (Fig. 9C, 10C, and 11C). Increasing concentrations of 5α-P resulted in a dose dependent trend towards alteration of arrangement of the actin cytoskeleton with fewer actin stress fibers occurring in cells treated with higher concentrations of 5αP (results not shown).

Treatment of cells with 3α-HP (10^{-10} M to 10^{-6} M) resulted in no apparent effect in organization of the F-actin stress fibre cytoskeleton (Fig. 9E and 9F respectively). Treatment of subconfluent and confluent cultures of MCF-7 cells with either 10^{-6} M or 10^{-10} M 3α-HP also resulted in no apparent differences in stress fiber organization from control cells (pictures not shown).

3.3.2 The Effect of 5αP and 3α-HP on Stress Fibre Numbers

Analysis of stress fibre numbers revealed that MCF-7 cells treated with 10^{-6} M 5α-P had significantly fewer (approximately 80%) stress fibres than either control cells, cells treated with 10^{-10} M 5α-P, or cells treated with 3α-HP ($p<0.01$, Fig. 12). Further analysis has shown that concentrations of 5α-P from 10^{-8}M to 10^{-6}M cause a significant dose dependent decrease in numbers of stress fibres (Fig.13). Concentrations of 5αP from 10^{-6}M to 10^{-5}M caused a consistent (80%) decrease in numbers of adhesion plaques (Fig. 13).

Treatment with 3α-HP at concentrations of 10^{-10} M and 10^{-6} did not cause a significant alteration in stress fibre numbers numbers relative to control cells (Fig.12). Treatment of cells with concentrations of 3α-HP between 10^{-9}M to 10^{-7}M showed a slight trend towards an increase in numbers of stress fibres, though the trend is not seen in at the lower concentration of 10^{-10}M and the higher concentrations 10^{-6}M to 10^{-5}M (Fig 13).

3.3.3 Quantification of Stress Fibre Fluorescence via Image Analysis

Digital computer image analysis of stress fibre fluorescence of Phalloidin-Rhodamine stained cells revealed that MCF-7 cells treated with 10^{-6} M 5α-P had significantly lower (approximately 45% of control) stress fibre fluorescence than either control cells, cells treated with 10^{-10} M 5α-P, or cells treated with 3α-HP ($p<0.01$, Fig. 14). Further analysis has shown that concentrations of 5α-P from 10^{-8}M to 10^{-7}M cause a significant dose dependant decrease in fluorescence of adhesion plaques (Fig. 15). Concentrations of 5αP from 10^{-6}M to 10^{-5}M caused a steady (approximately 60%) decrease in numbers of adhesion plaques (Fig. 15).

Treatment with 3α-HP at concentrations of 10^{-10} M and 10^{-6} did not cause a significant alteration or a trend in stress fibre fluorescence relative to control cells (Fig.14 and 15).

3.4 Actin Protein Expression

3.4.1 *Comparison of cellular concentrations of actin.*

Treatment of MCF-7 cells with 5α-P or 3α-HP at concentrations between 10^{-10}M and 10^{-5}M did not result in a significant change in the cellular concentration of actin (results not shown).

The cellular concentrations of monomeric G-actin and of polymeric F-actin were studied after separation of the two forms of actin using Triton X-100 (White *et al.*, 1983). Treatment of MCF-7 cells with 5α-P at concentrations greater than 10^{-9} M increased the cellular concentration of the monomeric G-actin and decreased the concentration of the polymeric F-actin fraction (Fig. 16). Treatment of MCF-7 cells with 3α-HP at concentrations between 10^{-10} M to 10^{-5} M did not result in an observable change in monomeric G-actin or polymeric F-actin (Fig. 16). Quantitative analysis of the effect of 5α-P on the degree of polymerization of the actin cytoskeleton of MCF-7 cells shows an increase in the ratio of G-actin vs F-actin at concentrations as low as 10^{-7} M (Fig. 17). No differences were seen in the ratio of G-actin:F-actin in cells treated with 3α-HP at any concentrations investigates (Fig. 17).

3.5 Vinculin Protein Expression

3.5.1 *Comparison of cellular concentrations of vinculin.*

Treatment of MCF-7 cells with 5α-P in concentrations greater than 10^{-9} M decreased the cellular concentration of vinculin while treatment of MCF-7 cells with concentrations of 3α-HP between 10^{-10}M and 10^{-5}M did not result in any change in the cellular concentration of vinculin (Fig. 18). Quantitative analysis of the effect of 5α-P at concentrations between 10^{10}M to 10^{-6}M on MCF-7 cells showed that 5αP caused a dose dependent decrease of in the amount of vinculin expressed (Fig. 19). Treatment of MCF-7 cells with concentrations of 5αP from 10^{-6}M to 10^{-5}M showed an approximate 75% decrease in levels of vinculin expressed (Fig. 19). No significant change was seen in cells treated with 3α-HP relative to control, though a slight trend towards an increase in vinculin expression was observed at higher concentrations (Fig. 19).

3.6 The Effects of 5αP and 3α-HP on Cell Morphology

Treatment of MCF-7 cells with 5α-P had a pronounced effect on cell morphology, while treatment with 3α-HP did not seem to alter cell morphology. Low magnification pictures of control cells (Fig. 20A and 20B) show a flat, circular morphology with few extending cellular processes (such as filopodia and lamelipodia) and continuous contact with adjacent cells. Cells treated with 10^{-6} M 5α-P (Fig. 20C and 20D) showed noticeable retraction of the cellular cytoplasm, leaving behind lamelipodia-like and filopodia-like processes. Cell-to-cell contacts were discontinuous in 5α-P treated cells, and were usually seen as point contacts via cellular microspikes or small filopodia-like structures. Treatment of MCF-7 cells with lower (10^{-9} M to 10^{-7} M) concentrations of 5αP resulted in a dose dependant decrease of retraction of the cytoplasm, with concentrations lower than 10^{-9} M showing no alterations in cellular morphology. Cells treated with 3α-HP did not show a visible change in morphology from control cells (Fig. 20E and 20F), possessing a flat, circular morphology with few cellular protrusions and continuous cell-to-cell contacts.

3.7 Morphometric Analysis

Cellular and nuclear size and shape, of the dozens of morphometric parameters that have been investigated, are the most commonly studied factors in morphometry. Cells treated with a sufficient concentration of 5α-P displayed a dose dependent decrease in cell area and a dose dependent increase in the irregularity of cell shape. Cells treated with a sufficient concentration of 5α-P also showed an increase in variability of nuclear area, though mean values of nuclear area and shape were not affected. Cells treated with 3α-HP did not display any alteration to cytoplasmic or nuclear area and shape.

3.7.1 Cellular Area and Shape

Morphometric analysis of cell area revealed that MCF-7 cells treated with 10^{-6} M 5α-P had a significantly smaller area (approximately 30%) than either control cells,

cells treated with 10^{-10} M 5α-P, or cells treated with 10^{-6} M or 10^{-10} M 3α-HP ($p<0.01$, Fig. 21). Increasing concentrations of 5α-P (10^{-8} M or 10^{-6}) resulted in a dose dependant decrease in cellular area (results not shown). Cells treated with 3α-HP did not exhibit a cellular area significantly different from control cells (Fig. 21).

Morphometric analysis of cell shape revealed that MCF-7 cells treated with 10^{-6} M 5α-P had a significantly more complex shape (approximately 25% more complex) than either control cells, cells treated with 10^{-10} M 5α-P, or cells treated with 3α-HP ($p<0.01$, Fig. 22). Increasing concentrations of 5α-P resulted in a dose dependant increase in cellular shape value (results not shown). Cells treated with 3α-HP did not exhibit a cellular shape significantly different from control cells (Fig. 22).

3.7.2 Nuclear Area and Nuclear Shape

Morphometric analysis of nuclear area reveals that MCF-7 cells treated with 5α-P or 3α-HP did not have a significantly different mean nuclear area than control cells (Fig. 23). Cells treated with 10^{-6} M 5α-P did have a significantly more variable nuclear area than either control cells, cells treated with 10^{-10} M 5α-P, or cells treated with 3α-HP ($p<0.01$, Fig. 23). Increasing concentrations of 5α-P resulted in a dose dependant increase in variability of nuclear area (results not shown). Cells treated with 3α-HP did not exhibit variability in nuclear area significantly different from control cells (Fig. 21).

Morphometric analysis of nuclear shape (nuclear perimeter relative to nuclear area) reveals that MCF-7 cells treated with 5α-P or 3α-HP did not have a significantly different mean 2-D nuclear area than control cells (results not shown).

3.8 Cytotoxicity of 5α-P and 3α-P

Treatment of MCF-7 cells with 5α-P or 3α-HP in concentrations greater than 10^{-4} M resulted in a significant increase in cellular mortality. Cells treated with 10^{-5} M 5α-P or 3α-HP did not show a significant difference from control, though a trend of greater mortality was observed (Appendix 2). Cells treated with concentrations of

3α-HP or 5α-P of 10^{-6}M or less did not show any change in mortality when compared to control.

Figure 1: The effects of 5α-P and 3α-HP on the detachment of subconfluent MCF-7 cells from the substrate. Cellular attachment was measured as described in *Materials and Methods*. Values are presented as mean ± SEM percentage MCF-7 cells detached. A significant increase in detachment occurs in cells treated with concentrations of 5α-P greater than 8×10^{-7} M, and a significant decrease in cellular detachment occurs in MCF7 cells treated with concentrations of 3α-HP greater than 4×10^{-7} M.

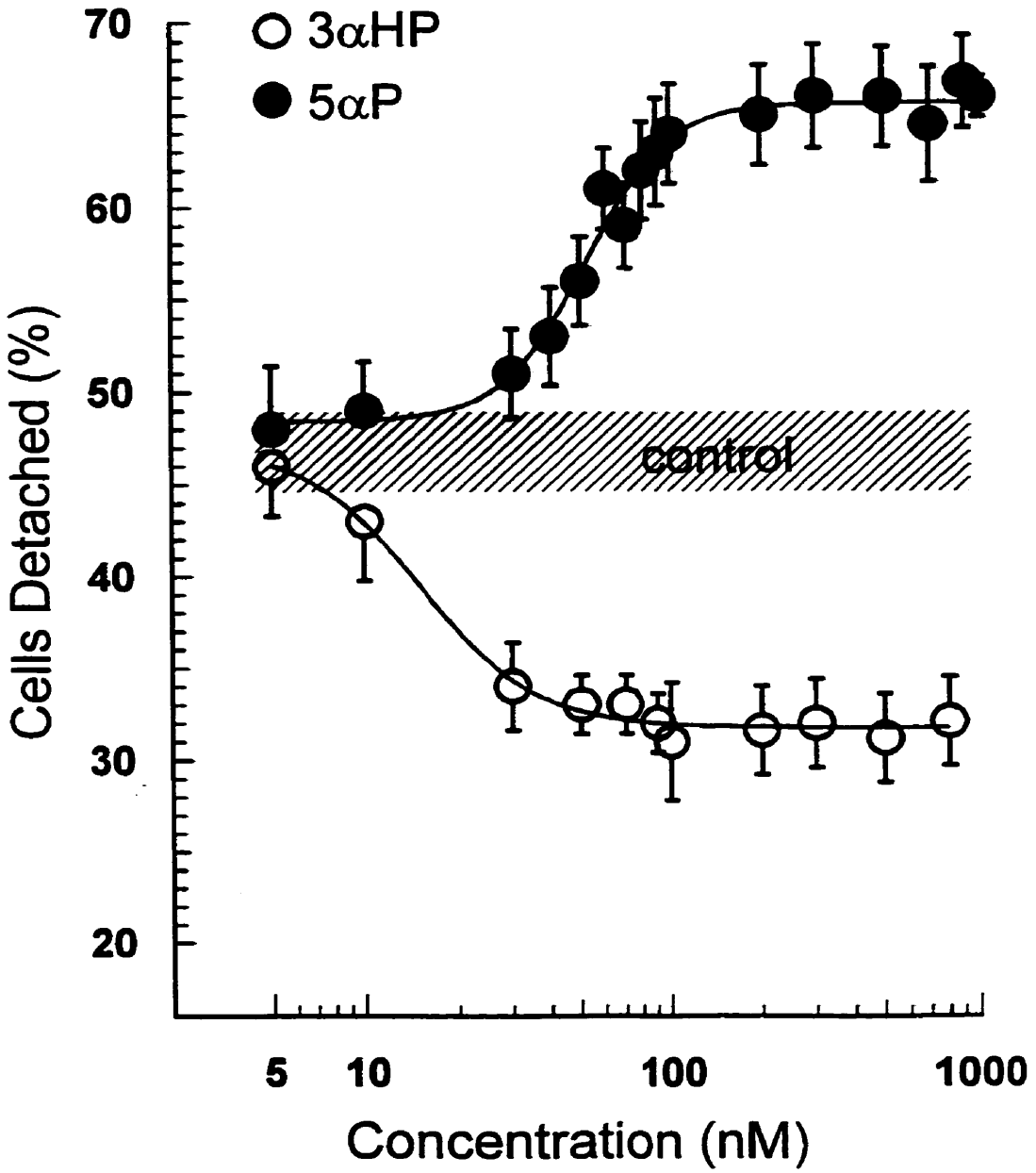

70
60
50
40
30
20
Cells Detached (%)
3αHP
5αP
control
5
10
100
1000
Concentration (nM)

Figure 2: The effects of 5α-P and 3α-HP on the attachment of subconfluent MCF-7 cells to the substrate. Cellular attachment was measured as described in *Materials and Methods*. Values are presented as mean ± SEM percentage MCF-7 cells attached. A significant decrease in attachment occurs in cells treated with concentrations of 5α-P greater than 7×10^{-7} M, and a significant increase in cellular attachment occurs in MCF-7 cells treated with concentrations of 3α-HP greater than 8×10^{-7} M.

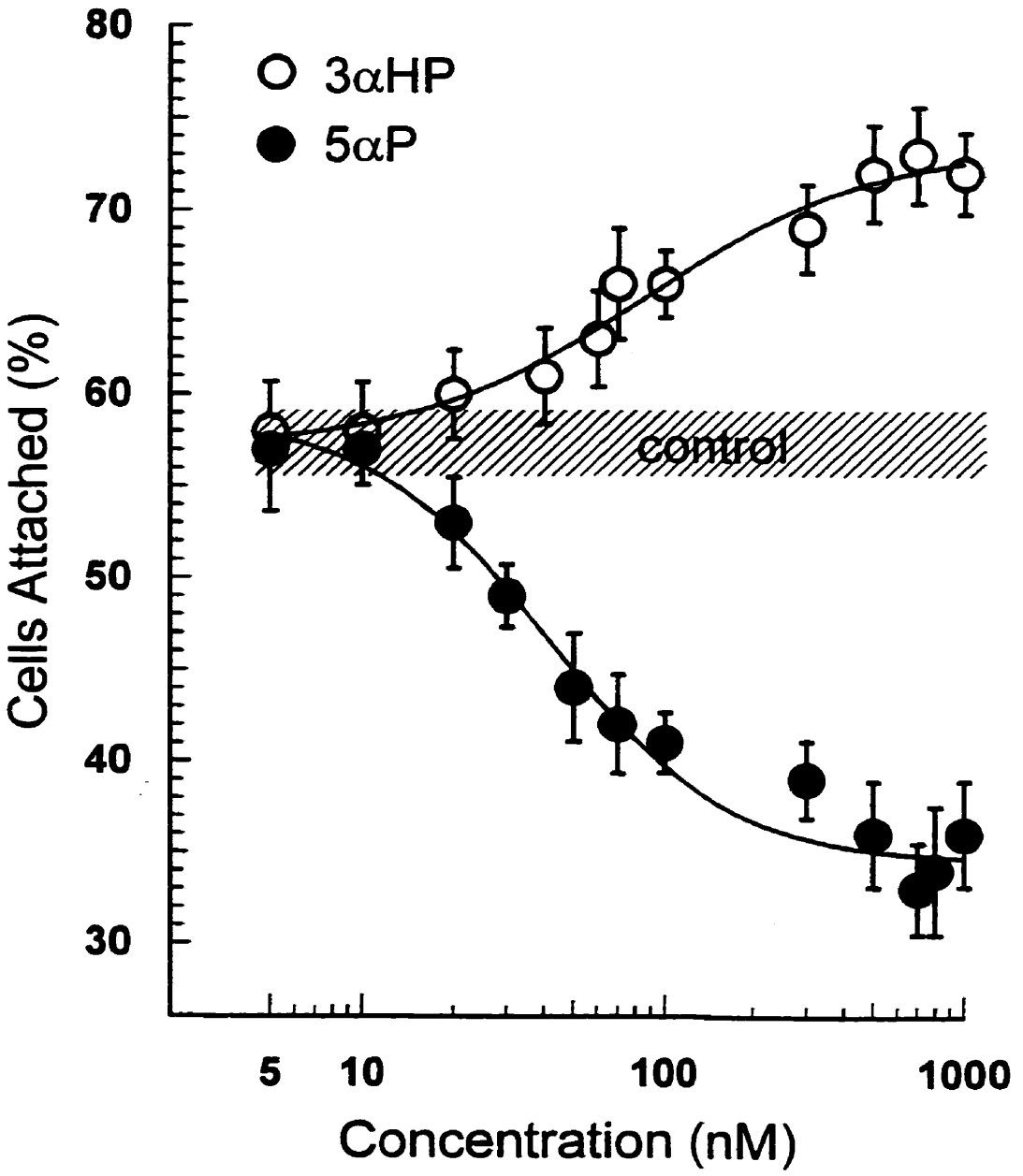

80
70
60
50
40
30
Cells Attached (%)
3αHP
5αP
control
5
10
100
1000
Concentration (nM)

Figure 3: **Representative photomicrographs of the effects of 5α-P and 3α-HP on the arrangement of vinculin containing adhesion plaques in single MCF-7 cells in culture.** Cells were grown for 3 days with no supplementary hormones (A and B), 10^{-10} M 5α-P (C), 10^{-6} M 5α-P (D), 10^{-10} M 3α-HP (E), and 10^{-6} M 3α-HP (F). Control cells (A and B), cells treated with 10^{-10} M 5α-P (C), and cells treated with 3α-HP (E and F) had numerous adhesion plaques (white arrows). Treatment with 10^{-6} M 5α-P (D) induces a large decrease in the amount of vinculin containing adhesion plaques within the cell. 320x.

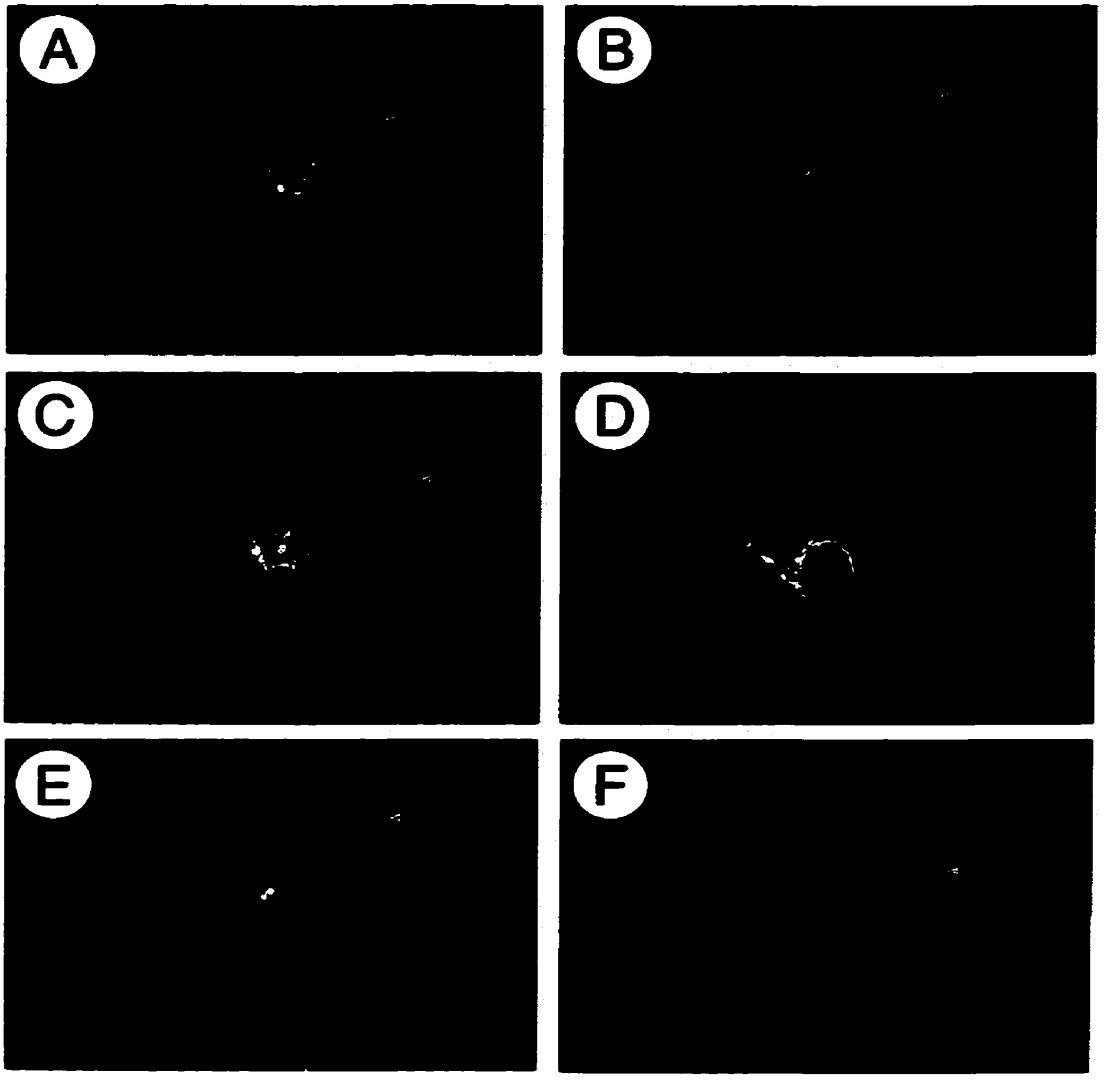

A
B
C
D
E
F

Figure 4: Representative photomicrographs of the effects of 5α-P and 3α-HP on the arrangement of vinculin containing adhesion plaques in confluent MCF-7 cells in culture. Cells were grown for 3 days with no supplementary hormones (A and B), 10^{-10} M 5α-P (C), 10^{-6} M 5α-P (D), 10^{-10} M 3α-HP (E), and 10^{-6} M 3α-HP (F). Examination of the photomicrographs reveals that treatments A, B, C, E, and F reveal numerous adhesion plaques, occurring both peripherally and basally (white arrows). Treatment with 10^{-6} M 5α-P (D) induces a large decrease in the amount of basal and peripheral vinculin containing adhesion plaques. 410x.

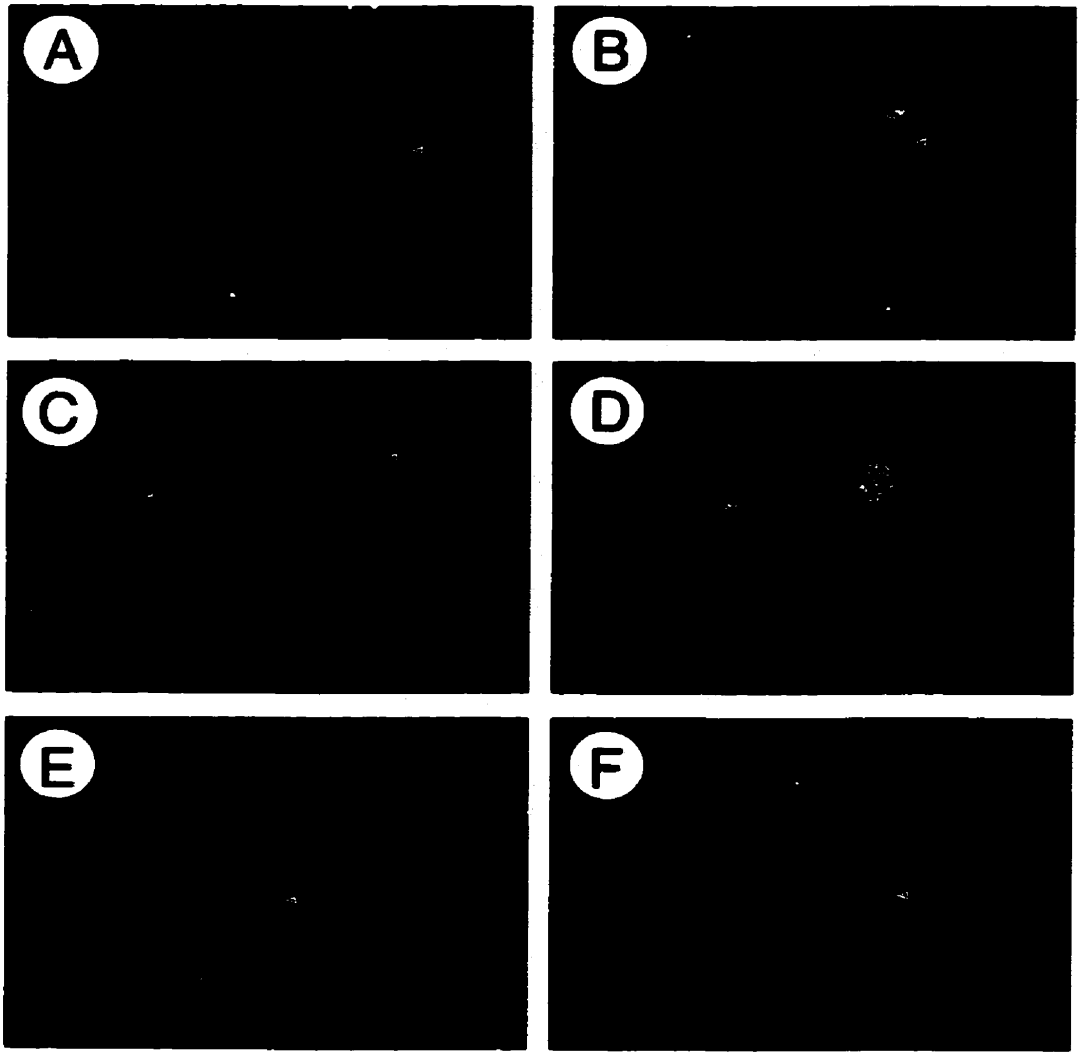

A
B
C
D
E
F

Figure 5: The effects of 5α-P and 3α-HP on the number of vinculin containing adhesion plaques within individual MCF-7 cells in culture. Values are presented as mean ± SEM (t-test, n=10 experiments, 45 cells/experiment) number of vinculin containing adhesion plaques per individual MCF-7 cell.

Within each treatment group, bars labeled with different letters are significantly different at $p<0.01$.

* significantly different at $p<0.01$ compared to control and 3α-HP treatment.

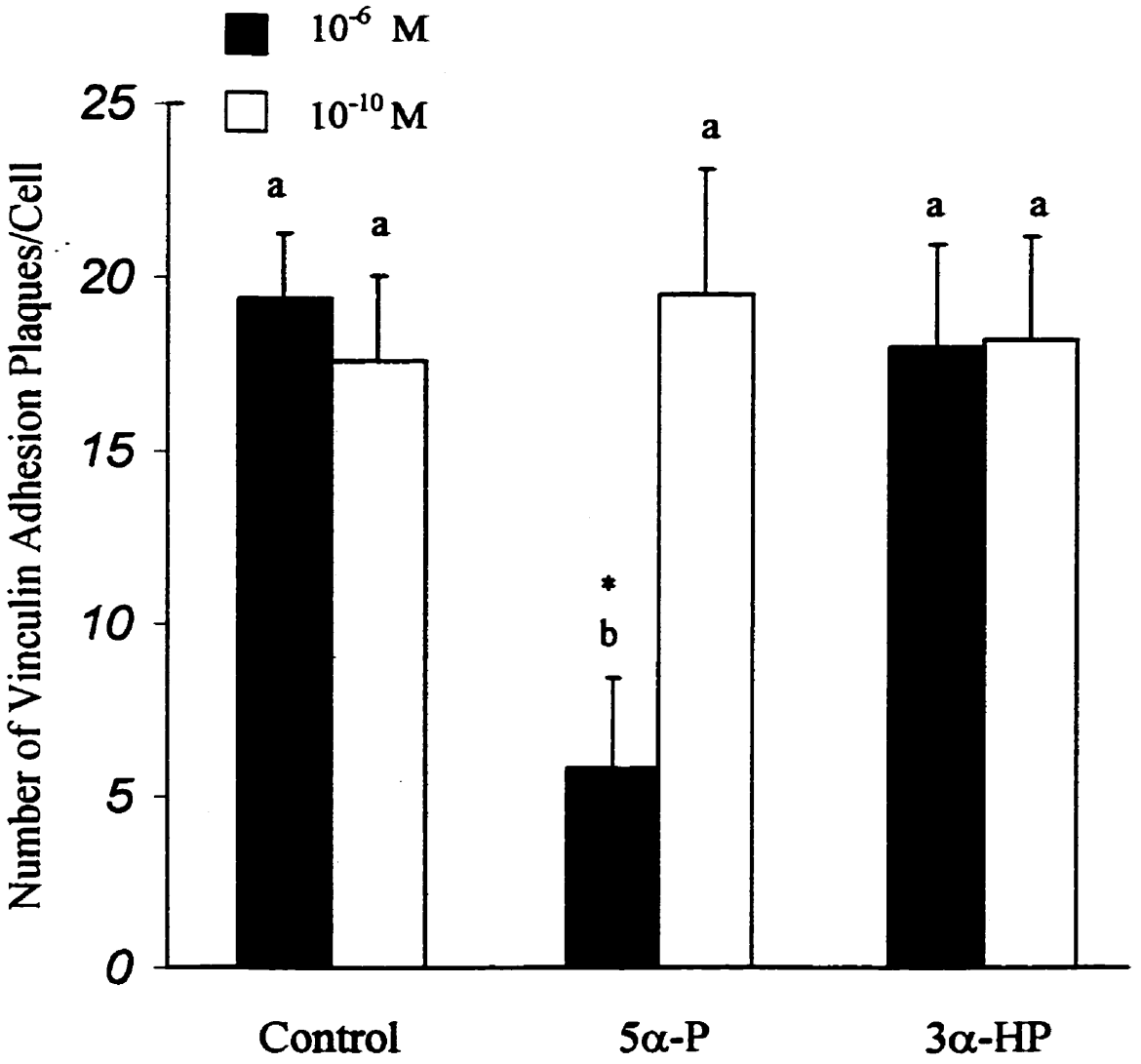

10^-6 M
10^-10 M
Number of Vinculin Adhesion Plaques/Cell
25
20
15
10
5
0
a
a
*
b
a
a
a
Control
5α-P
3α-HP

Figure 6: The dose dependent effects of 5α-P and 3α-HP on the number of vinculin containing adhesion plaques within MCF-7 cells in culture. Values are presented as mean ± SEM (t-test, n=10 experiments, 45 cells/experiment) number of vinculin containing adhesion plaques per individual MCF-7 cell.

* significantly different at $p<0.05$ compared to control.

** significantly different at $p<0.01$ compared to control.

Note: 3α-HP data points are offset to avoid overlapping symbols.

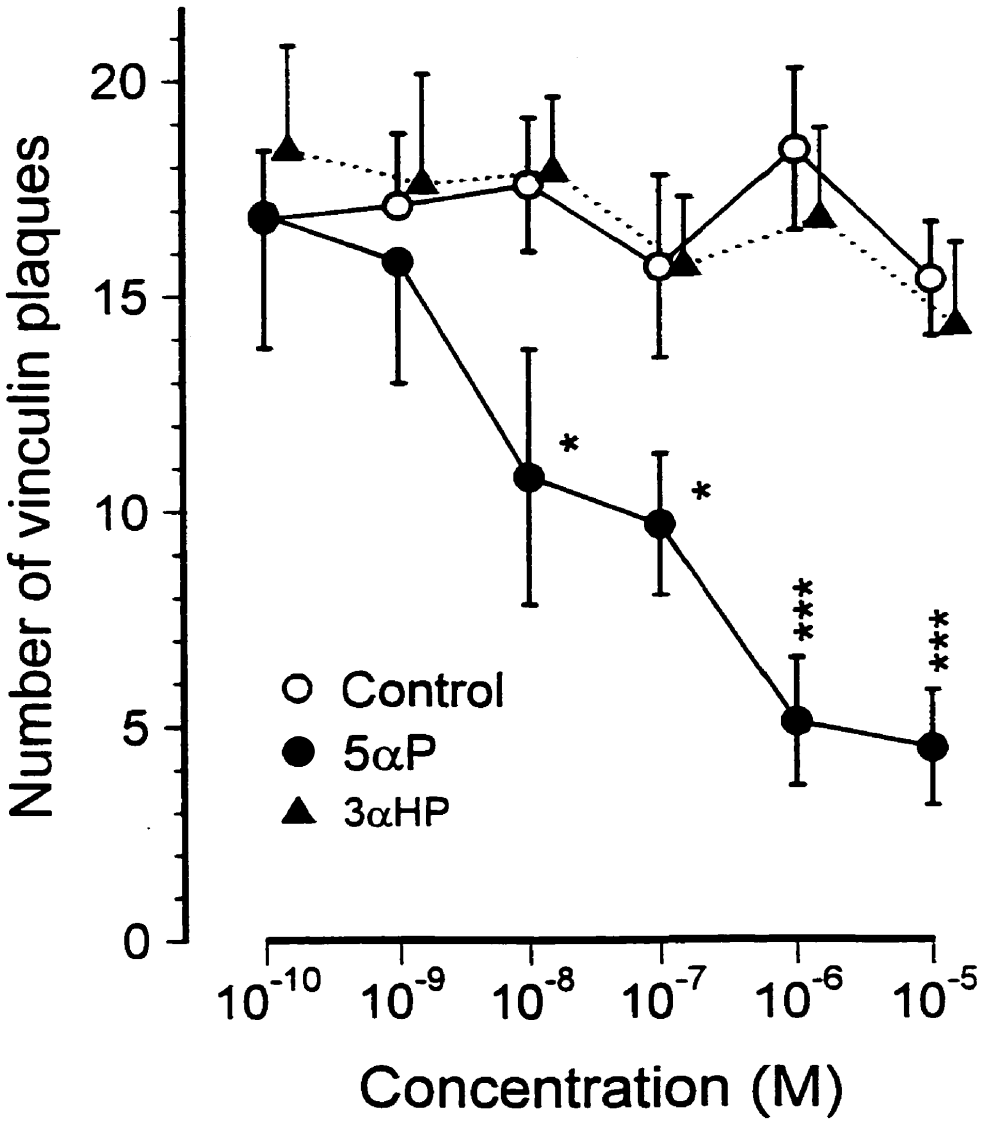

Number of vinculin plaques
20
15
10
5
0
Control
5αP
3αHP
*
*
***
***
10-10
10-9
10-8
10-7
10-6
10-5
Concentration (M)

Figure 7: The effects of 5α-P and 3α-HP on the fluorescence of subconfluent MCF-7 cells stained with mAb to human vinculin. Optical brightness was quantified by computer image analysis, as described in *Materials and Methods*. Values are presented as mean ± SEM (t-test, n=10 experiments, 150 cells/experiment) fluorescence/cell of individual MCF-7 cells.

Within each treatment group, bars labeled with different letters are significantly different at $p<0.01$.

* significantly different at $p<0.01$ compared to control and 3α-HP treatment.

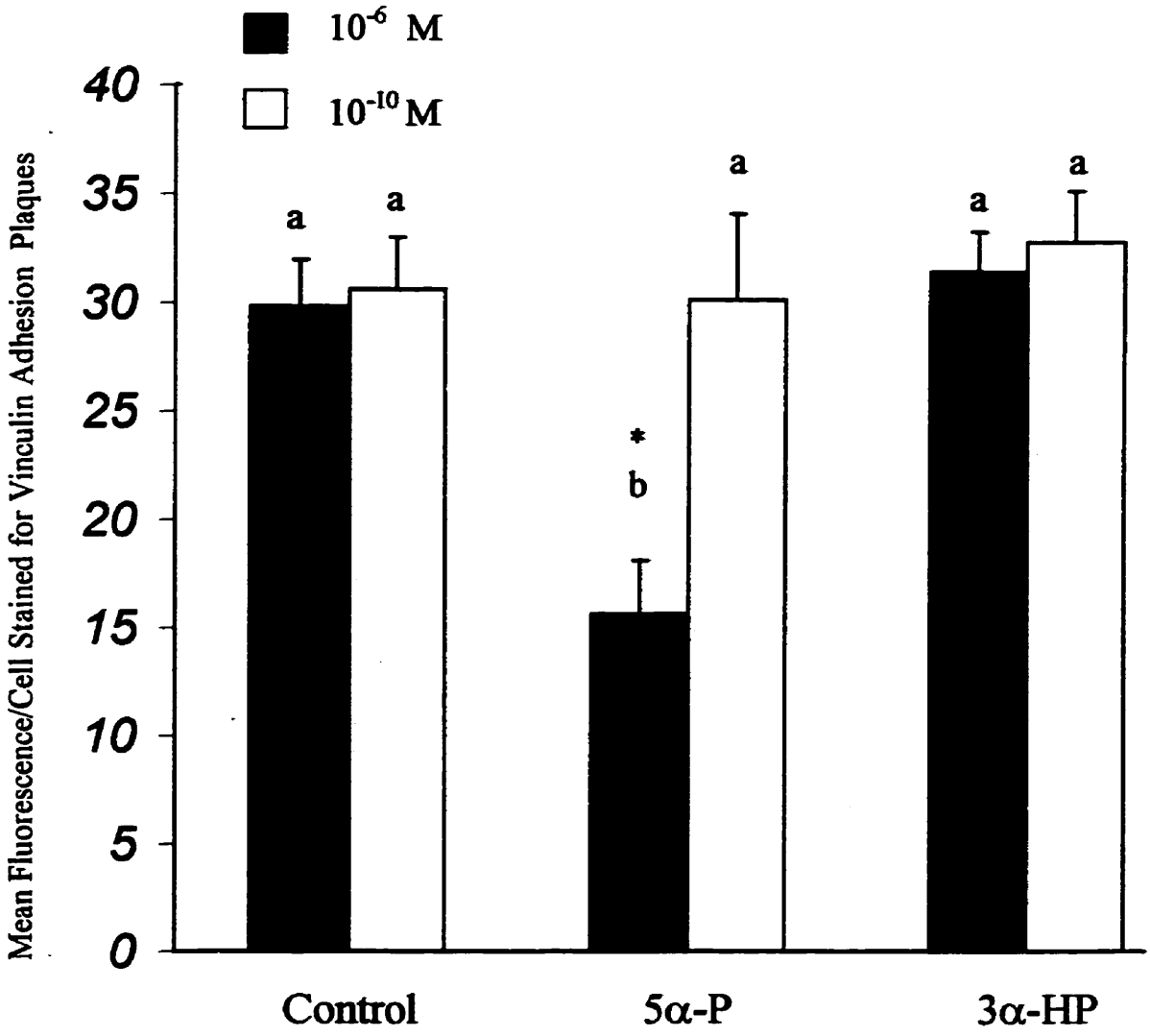

10-6 M
10-10 M
Mean Fluorescence/Cell Stained for Vinculin Adhesion Plaques
40
35
30
25
20
15
10
5
0
a
a
*
b
a
a
a
Control
5α-P
3α-HP

Figure 8: The dose dependent effects of 5α-P and 3α-HP on the fluorescence of subconfluent MCF-7 cells stained with mAb to human vinculin. Fluorescence was quantified by computer image analysis, as described in *Materials and Methods*. Values are presented as mean ± SEM (t-test, n=10 experiments, 150 cells/experiment) fluorescence/cell of individual MCF-7 cells.

* significantly different at $p<0.05$ compared to control.

*** significantly different at $p<0.001$ compared to control.

Note: 3α-HP data points are slightly offset to avoid overlapping symbols.

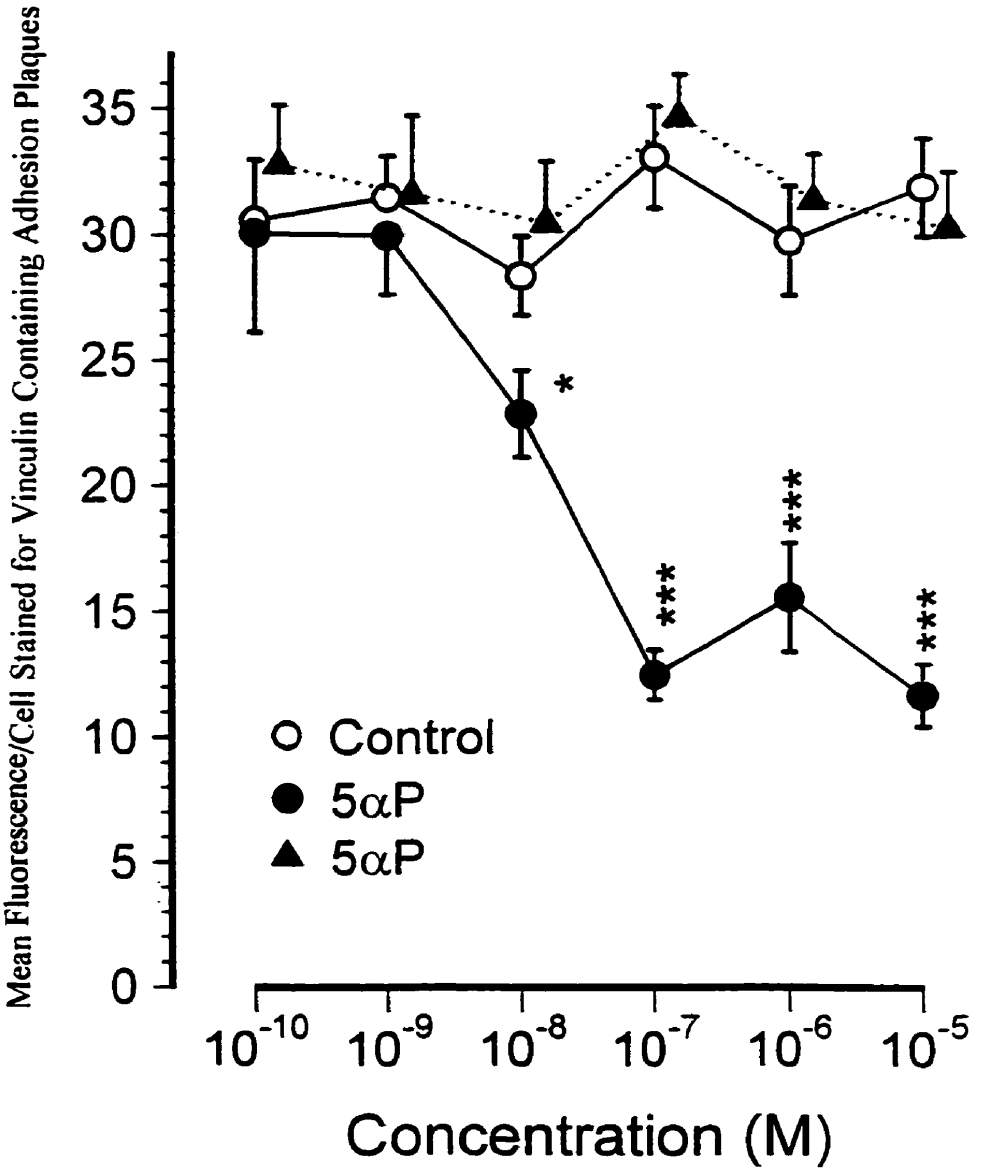

Mean Fluorescence/Cell Stained for Vinculin Containing Adhesion Plaques
35
30
25
20
15
10
5
0
*
***
***
***
Control
5αP
5αP
10-10
10-9
10-8
10-7
10-6
10-5
Concentration (M)

Figure 9: Representative photomicrographs of the effects of 5α-P and 3α-HP on the F-actin stress fiber cytoskeleton in single MCF-7 cells in culture. Cells were grown for 3 days with no supplementary hormones (A and B), 10^{-10} M 5α-P (C), 10^{-6} M 5α-P (D), 10^{-10} M 3α-HP (E), and 10^{-6} M 3α-HP (F). Examination of the photomicrographs reveals that treatments A, B, C, E, and F reveal a complex interconnecting network of stress fibers (white arrows). Treatment with 10^{-6} M 5α-P (D) induces a large decrease in the amount and complexity of the actin stress fiber cytoskeleton within the cell. 360x.

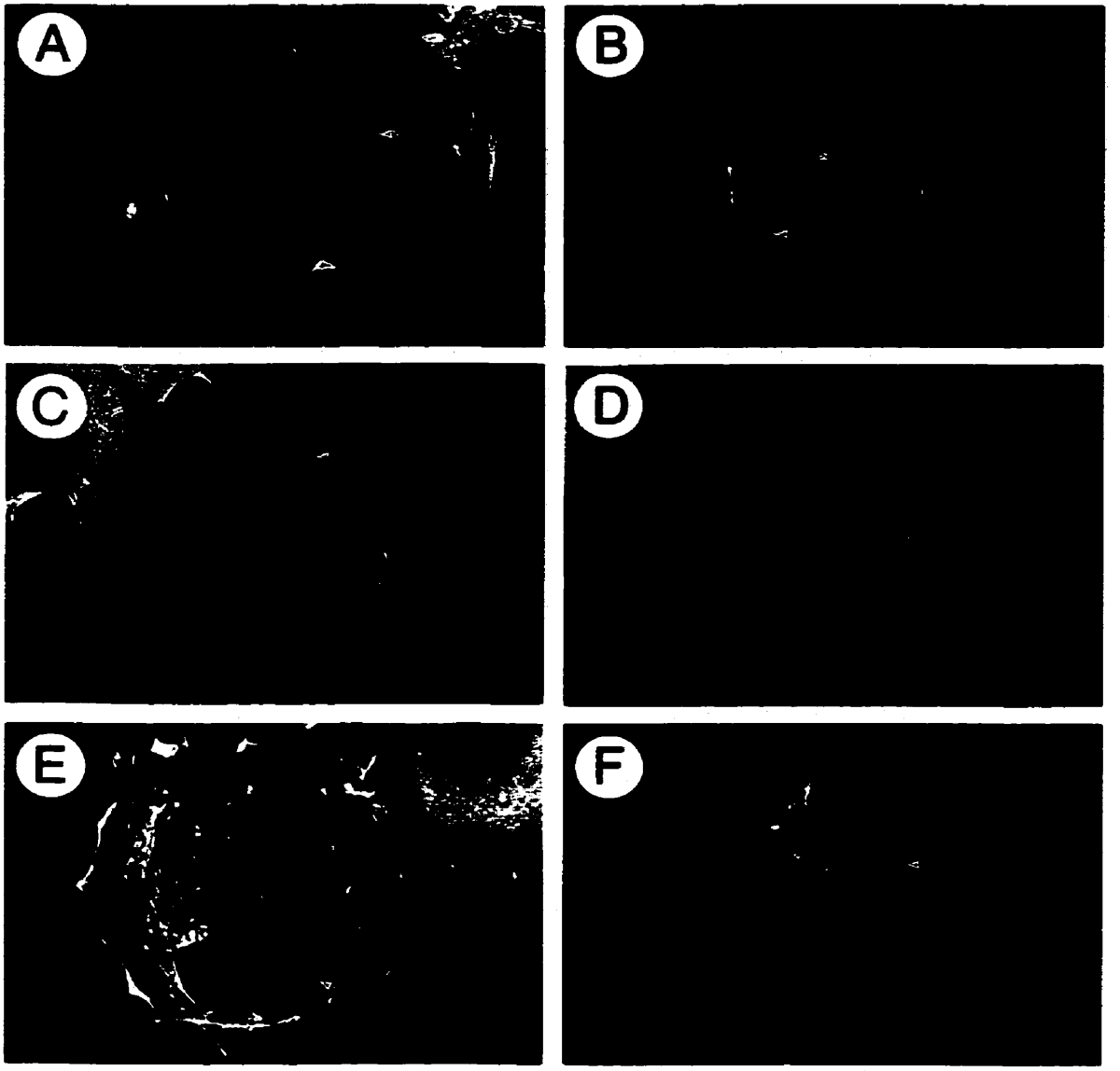

A
B
C
D
E
F

Figure 10: Representative photomicrographs of the effects of 5α-P and 3α-HP on the F-actin stress fiber cytoskeleton in subconfluent MCF-7 cells in culture. Cells were grown for 3 days with no supplementary hormones (A and B), 10^{-10} M 5α-P (C), and 10^{-6} M 5α-P (D). Examination of the photomicrographs reveals that treatments A, B, and C, reveal a complex interconnecting network of stress fibers (white arrows), and continuous cell-to-cell contacts. Treatment with 10^{-6} M 5α-P (D) induces a large decrease in the amount and complexity of the actin stress fibers cytoskeleton within the cell and discontinuous cell-to-cell contacts between cells. Treatment with 10^{-6} M and 10^{-10} M 3α-HP did not show any difference from control cells (pictures not shown). 460x.

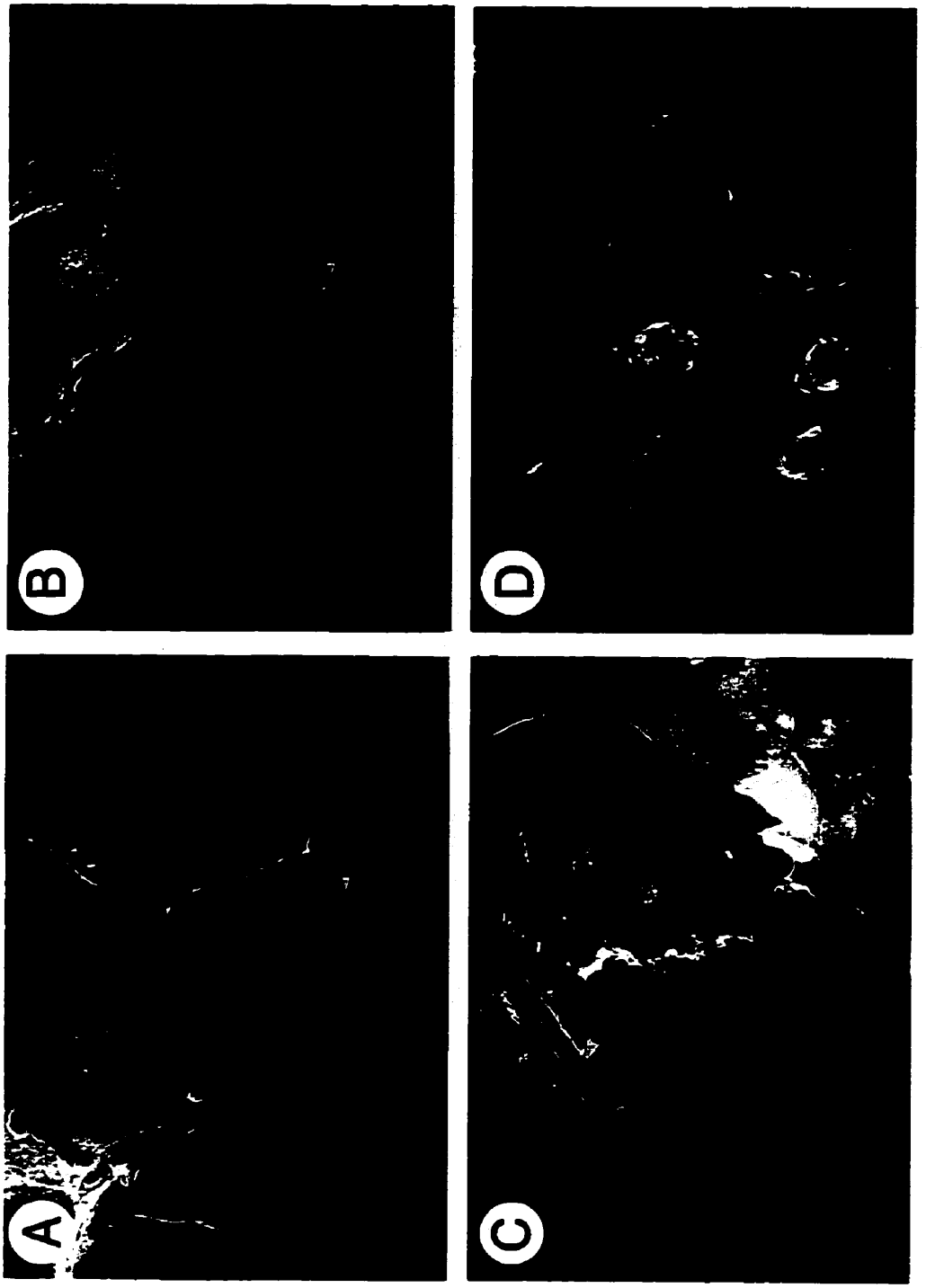

B
D
A
C

Figure 11: Representative photomicrographs of the effects of 5α-P and 3α-HP on the F-actin stress fiber cytoskeleton in confluent MCF-7 cells in culture. Cells were grown for 3 days with no supplementary hormones (A and B), 10^{-10} M 5α-P (C), 10^{-6} M 5α-P (D). Note: Examination of the photomicrographs reveals that treatments A, B, and C reveal a complex interconnecting network of stress fibers (white arrows), and continuous cell-to-cell contacts. Treatment with 10^{-6} M 5α-P (D) induces a large decrease in the amount and complexity of the actin stress fibers cytoskeleton within the cell and discontinuous cell-to-cell contacts between cells. Treatment with 10^{-6} M and 10^{-10} M 3α-HP did not show any difference from control cells (pictures not shown). 420x.

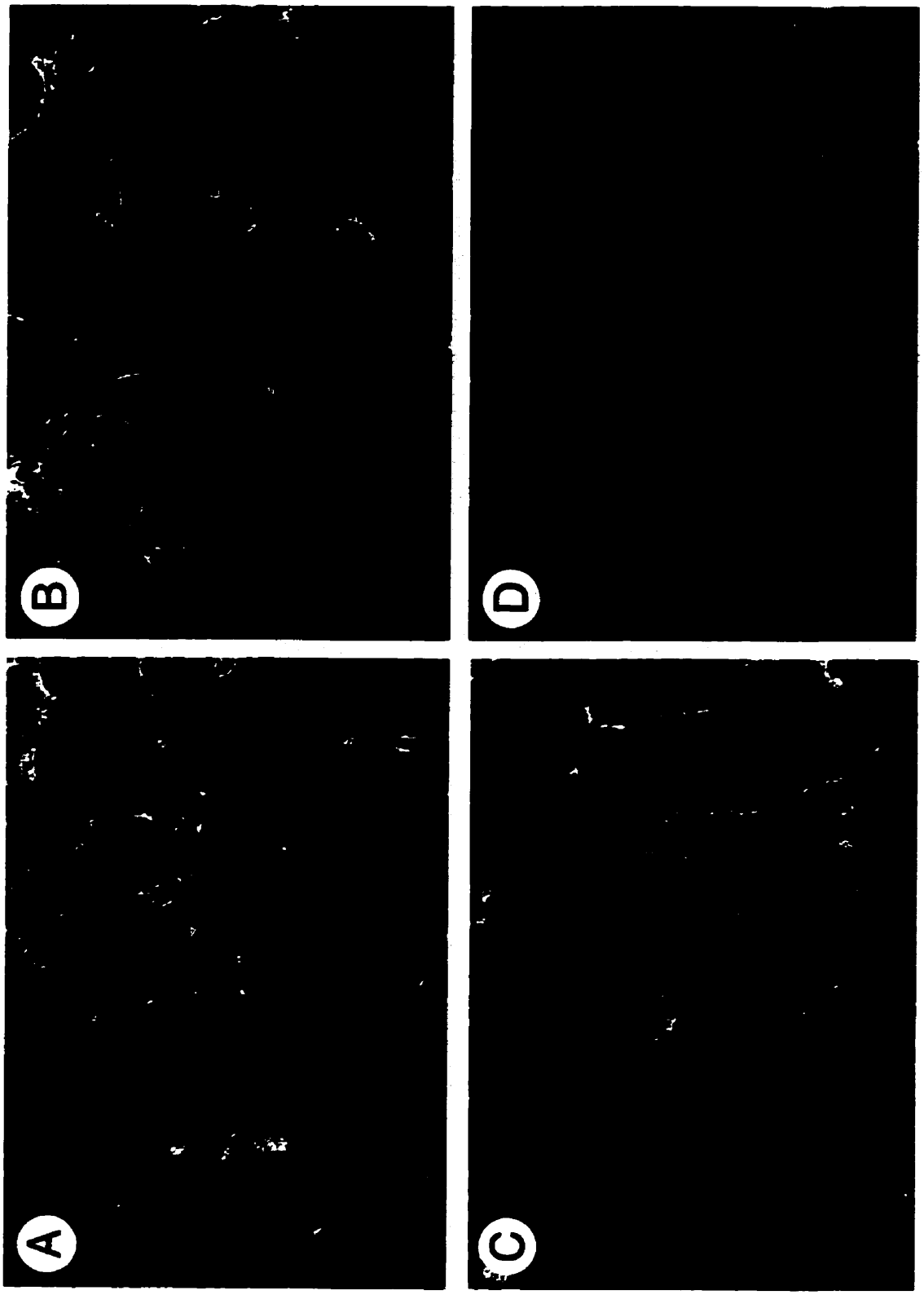

B
D
A
C

Figure 12: The effects of 5α-P and 3α-HP on the number of actin stress fibers within individual MCF-7 cells in culture. Values are presented as mean ± SEM (t-test, n=10 experiments, 45 cells/experiment) number of actin stress fibers per individual MCF-7 cell.

Within each treatment group, bars labeled with different letters are significantly different at $p<0.01$.

* significantly different at $p<0.01$ compared to control and 3α-HP treatment.

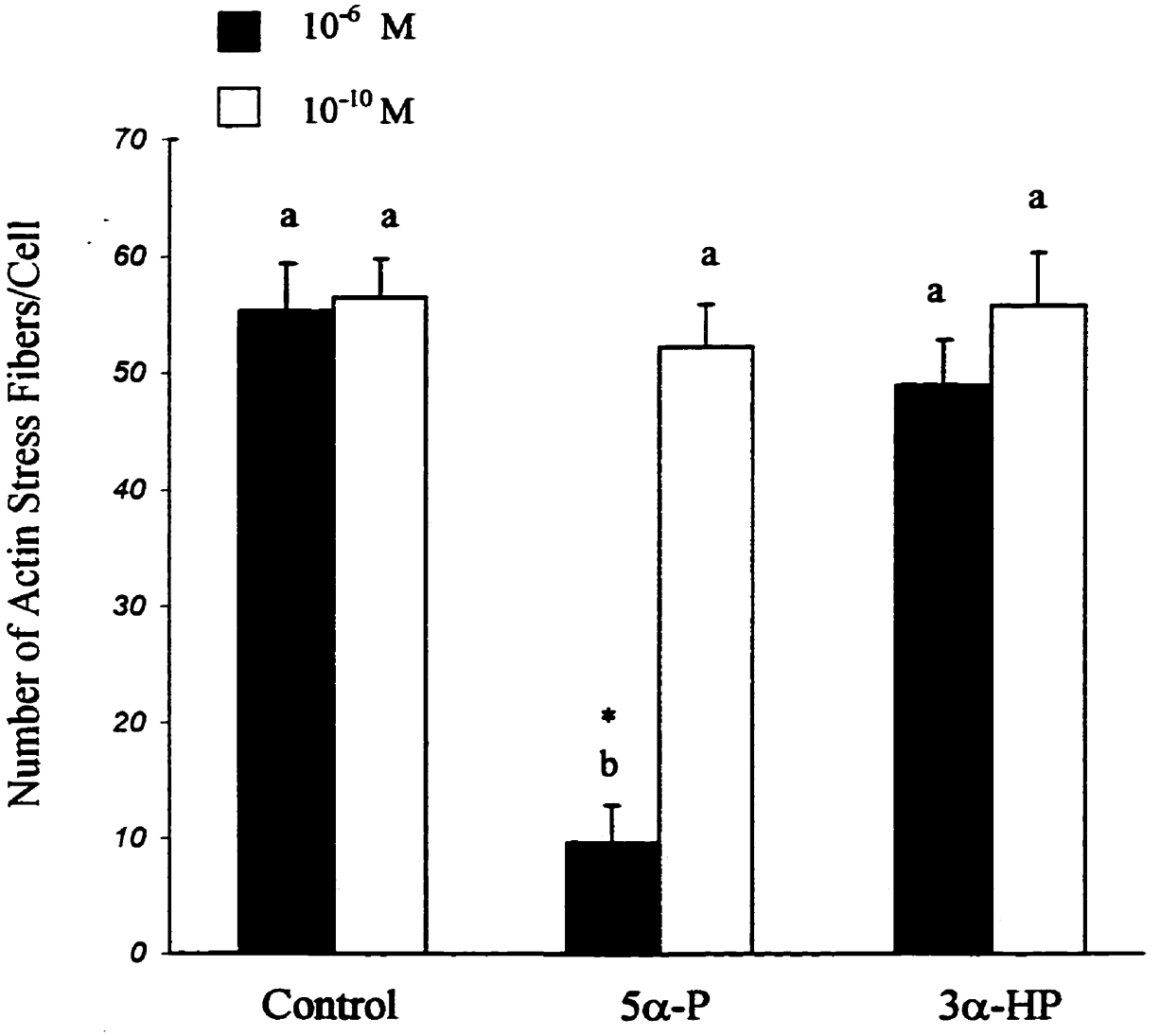

10-6 M
10-10 M
Number of Actin Stress Fibers/Cell
70
60
50
40
30
20
10
0
a
a
a
a
a
*
b
Control
5α-P
3α-HP

Figure 13: The dose dependent effects of 5α-P and 3α-HP on the number of actin stress fibers within individual MCF-7 cells in culture. Values are presented as mean ± SEM (t-test, n=10 experiments, 45 cell/experiment) number of actin stress fibers per individual MCF-7 cell.

* significantly different at $p<0.05$ compared to control.

** significantly different at $p<0.01$ compared to control.

Note: 3α-HP data points are slightly offset to avoid overlapping symbols.

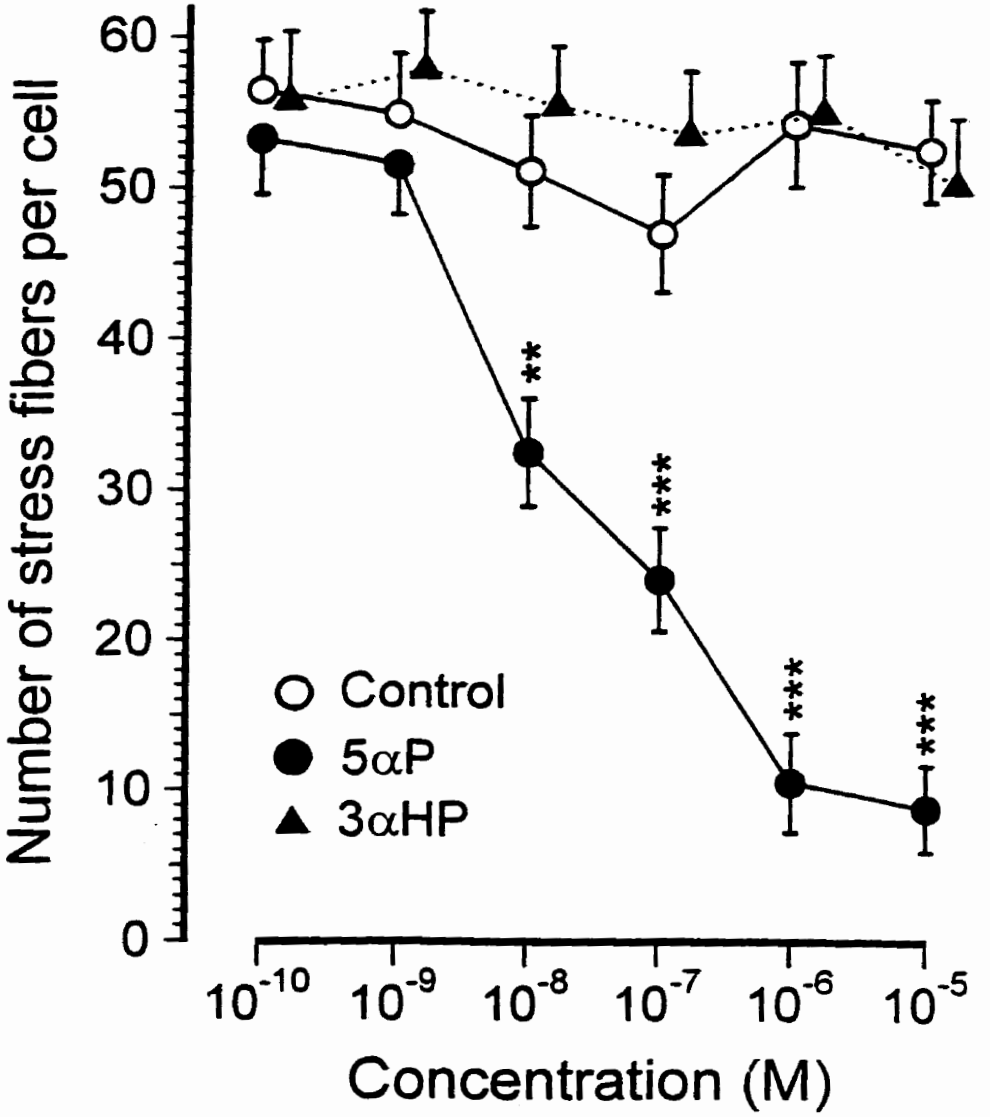

Number of stress fibers per cell
60
50
40
30
20
10
0
Control
5αP
3αHP
**
***
***
***
10-10
10-9
10-8
10-7
10-6
10-5
Concentration (M)

Figure 14: The effects of 5α-P and 3α-HP on the fluorescence of subconfluent MCF-7 cells stained with Rhodamine-Phalloidin. Fluorescence was quantified by computer image analysis, as described in *Materials and Methods*. Values are presented as mean ± SEM (t-test, n=10 experiments, 150 cells/experiment) fluorescence/cell of individual MCF-7 cells and are

Within each treatment group, bars labeled with different letters are significantly different at $p<0.01$.

* significantly different at $p<0.01$ compared to control and 3α-HP treatment.

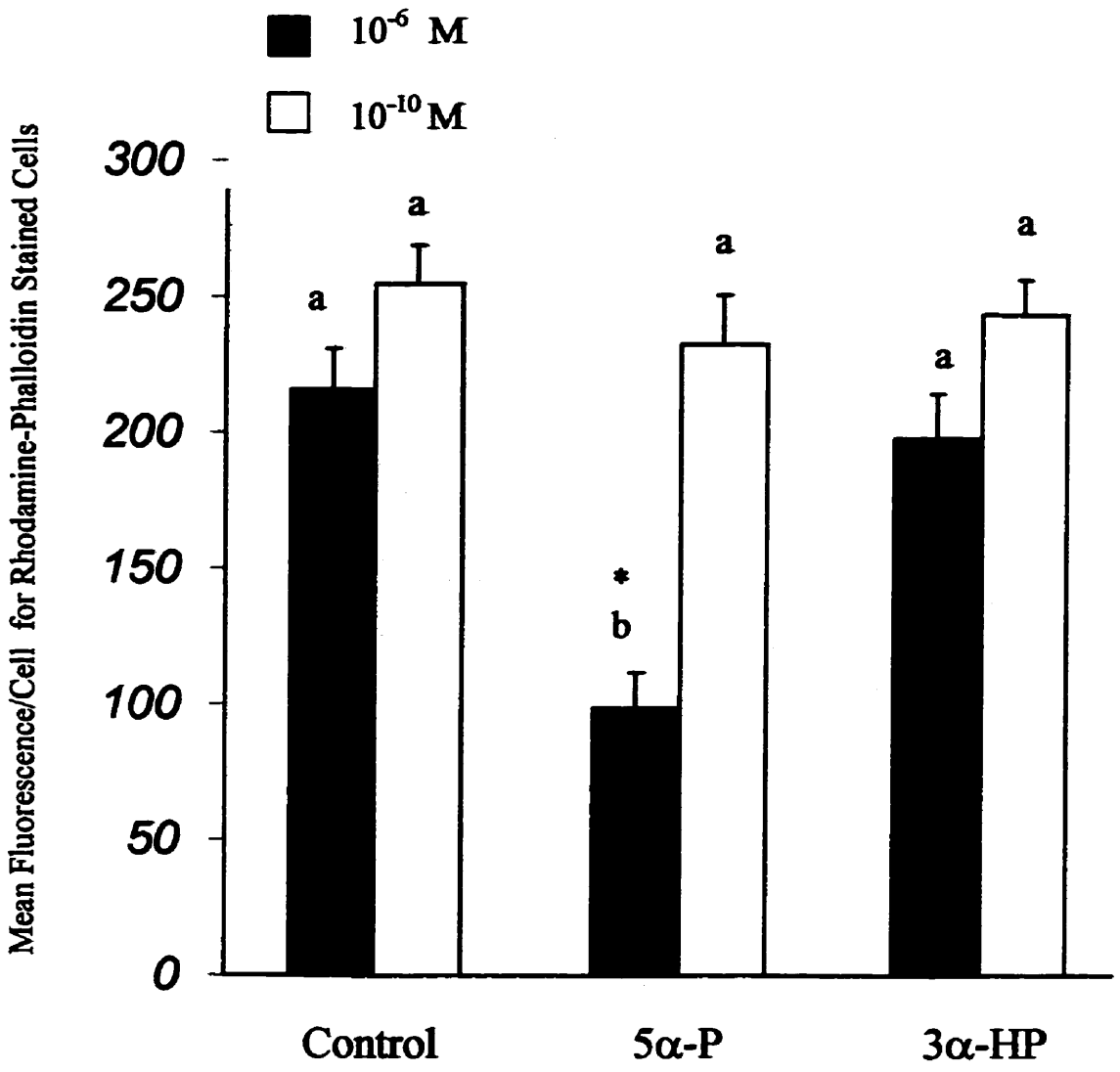

10-6 M
10-10 M
Mean Fluorescence/Cell for Rhodamine-Phalloidin Stained Cells
300
250
200
150
100
50
0
a
a
*
b
a
a
a
a
Control
5α-P
3α-HP

Figure 15: The dose dependent effects of 5α-P and 3α-HP on the fluorescence of MCF-7 cells stained with Rhodamine-Phalloidin. Fluorescence was quantified by computer image analysis, as described in *Materials and Methods*. Values are presented as mean ± SEM (t-test, n=10 experiments, 150 cells/experiment) fluorescence/cell of individual MCF-7 cells.

* significantly different at $p<0.01$ compared to control.

** significantly different at $p<0.01$ compared to control.

Note: 3α-HP symbols data points are slightly offset to avoid overlapping symbols.

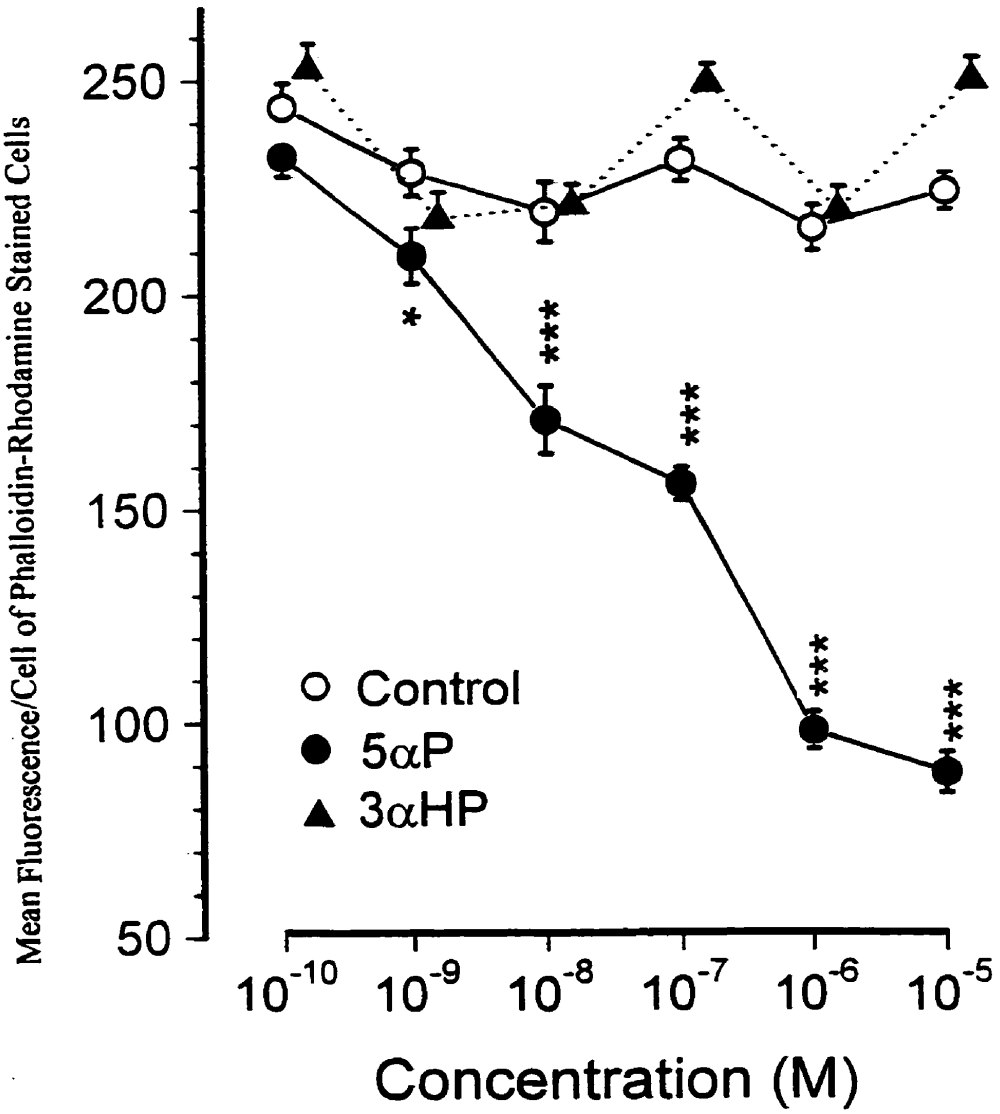

Mean Fluorescence/Cell of Phalloidin-Rhodamine Stained Cells
250
200
150
100
50
Control
5αP
3αHP
*
***
***
***
***
10^-10
10^-9
10^-8
10^-7
10^-6
10^-5
Concentration (M)

Figure 16: The effects of 5α-P and 3α-HP on the relative amounts of soluble and insoluble actin within subconfluent MCF-7 cells in culture. Soluble and insoluble actin fractions were collected, sorted via SDS-PAGE, and transferred via Western Blotting as described in *Materials and Methods*. Proteins detected were actin (treatment) or ornithine decarboxylase (control). Fractions consist of Soluble/Control (Lane 1), Insoluble/Control (Lane 2), Soluble/5α-P (Lane 3), Insoluble/5α-P (Lane 4), Soluble/3α-HP (Lane 5), and Insoluble/3α-HP (Lane 6). Note: There is no difference between soluble and insoluble actin fractions of control and 3α-HP treated MCF-7 cells at any hormone concentration. There is a visible increase in the amount of soluble actin and a decrease in the amount of insoluble actin in 5α-P treated cells in hormone concentrations from 10^{-5} M to 10^{-7} M relative to the control.

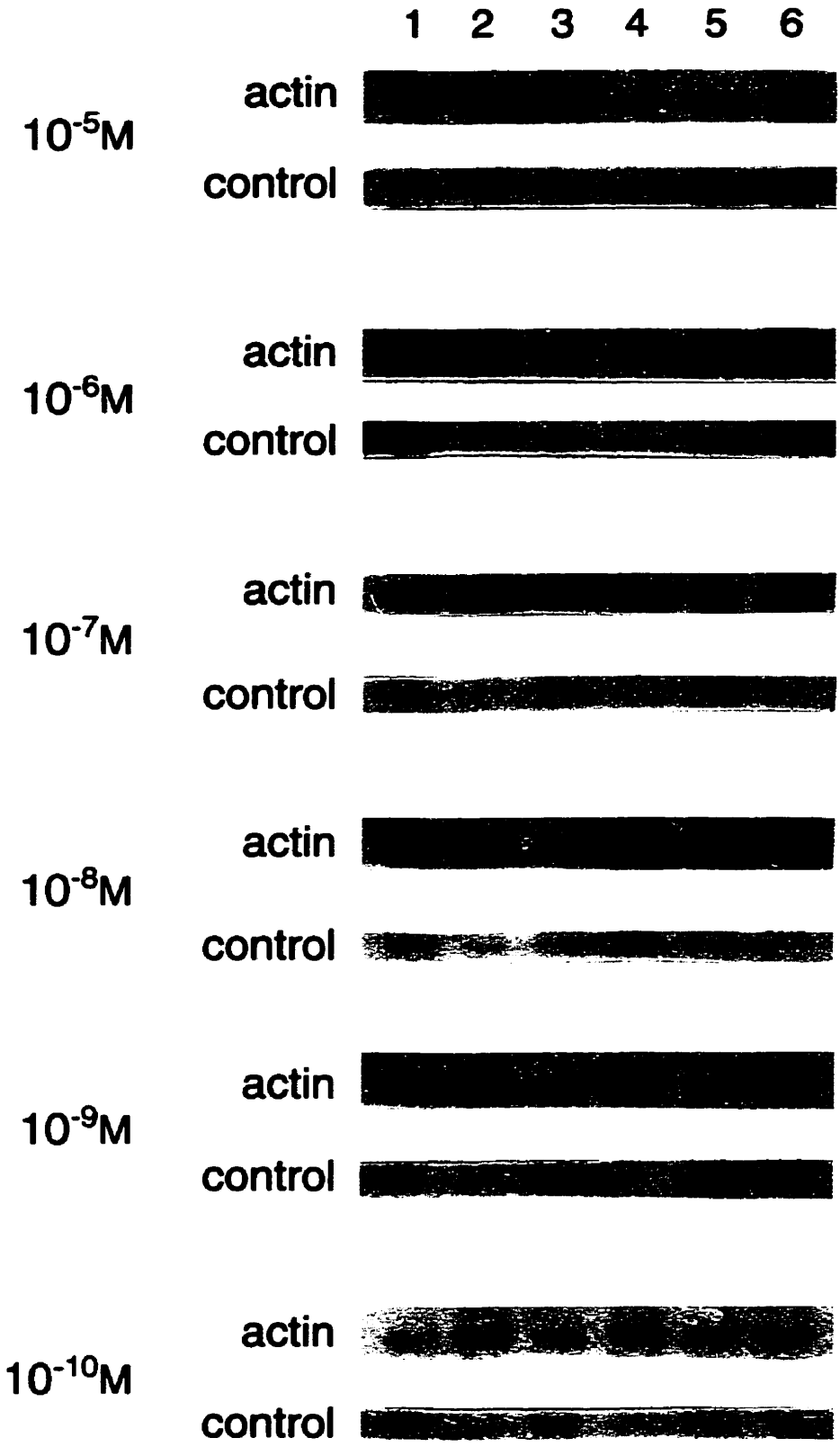

1 2 3 4 5 6
actin
10^{-5}M
control
actin
10^{-6}M
control
actin
10^{-7}M
control
actin
10^{-8}M
control
actin
10^{-9}M
control
actin
10^{-10}M
control

Figure 17: The effect of 5α-P and 3α-HP treatment on the optical density ratio of western blot bands of soluble (G-actin):insoluble (F-actin) within MCF-7 cells in culture. Soluble and insoluble actin fractions were collected, sorted via SDS-PAGE, transferred via Western Blotting, and their optical densities determined as described in *Materials and Methods*. Data presented as mean optical density (n=5). Note: There is a decrease in the ratio of soluble actin:insoluble actin in 5α-P treated cells in hormone concentrations from 10^{-8} M to 10^{-5} M relative to the control.

Note: 3α-HP data points are slightly offset to avoid overlapping symbols.

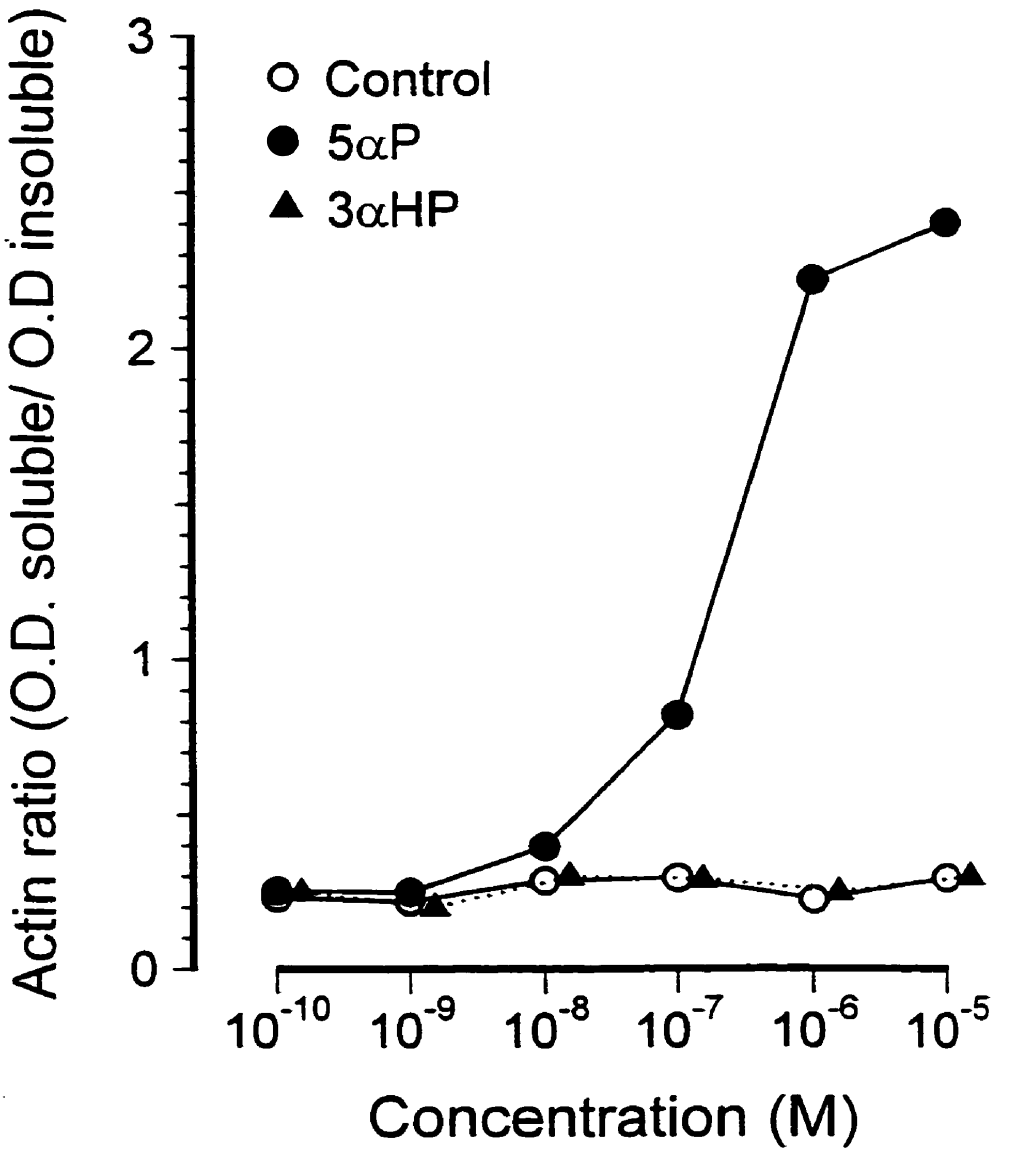

Actin ratio (O.D. soluble/ O.D insoluble)
3
2
1
0
Control
5αP
3αHP
10-10
10-9
10-8
10-7
10-6
10-5
Concentration (M)

Figure 18: The effects of 5α-P and 3α-HP on the expression of vinculin within subconfluent MCF-7 cells in culture. Cellular proteins were sorted via SDS-PAGE, and transferred via Western Blotting as described in *Materials and Methods*. Proteins detected were actin (treatment) or ornithine decarboxylase (control). Cells were grown in hormone concentrations of 10^{-5} M (block A), 10^{-6} M (block B), 10^{-7} M (block C), 10^{-8} M (block D), 10^{-9} M (block E), and 10^{-10} M (block F). Fractions consist of Control (Lane 1), 5α-P (Lane 2), and 3α-HP (Lane 3). There is no significant difference in vinculin expression between control and 3α-HP treated MCF-7 cells at any hormone concentration. There is a decrease in the amount of vinculin in 5α-P treated cells in hormone concentrations from 10^{-8} M to 10^{-5} M relative to the control.

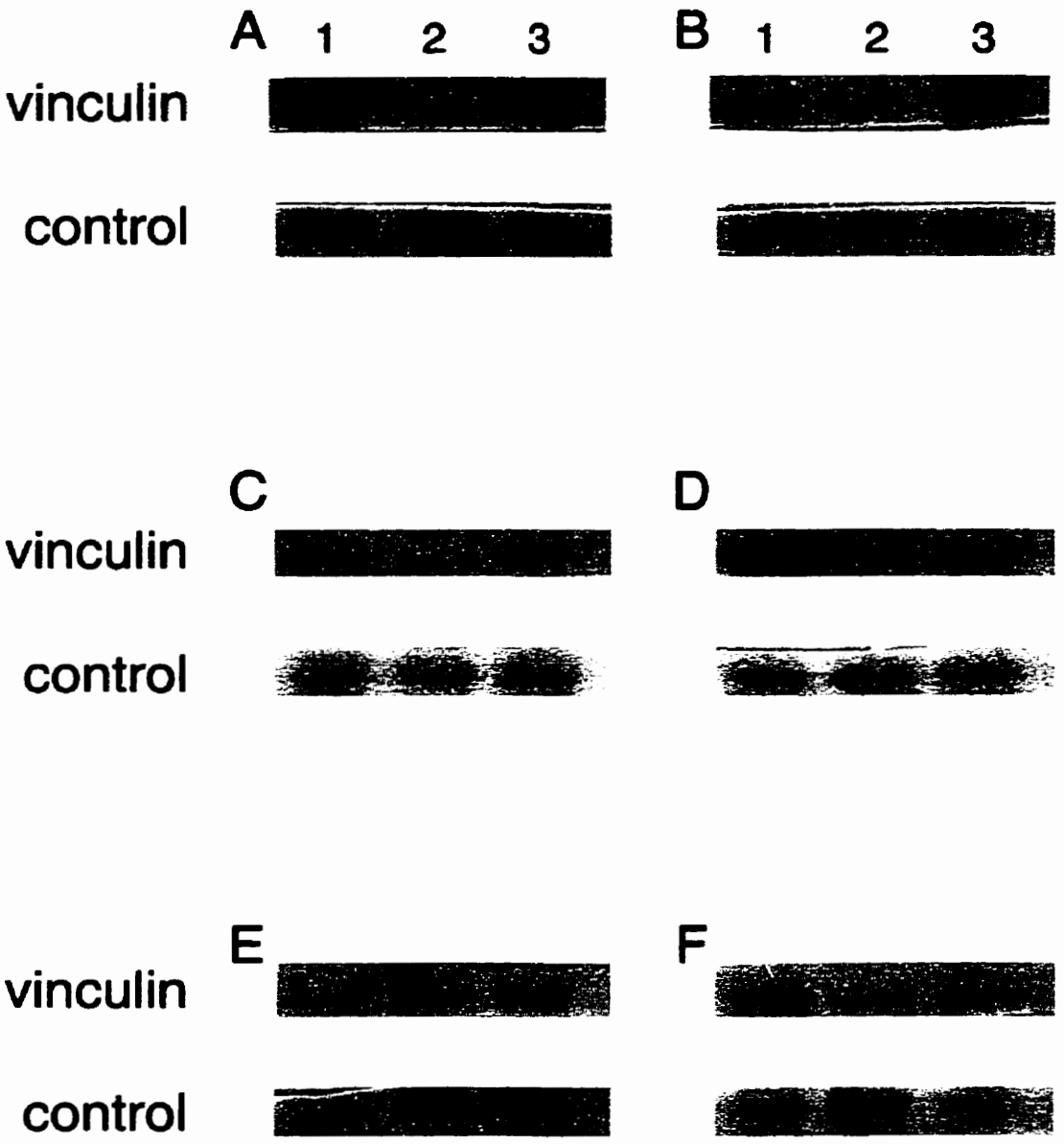

A
1
2
3
B
1
2
3
vinculin
control
C
D
vinculin
control
E
F
vinculin
control

Figure 19: The effect of 5α-P and 3α-HP treatment on the optical density of western blot bands of vinculin within subconfluent MCF-7 cells in culture. Vinculin containing fractions were collected, sorted via SDS-PAGE, and transferred via Western Blotting, and their optical densities determined as described in *Materials and Methods*. Note: There is a significant decrease in the amount of vinculin in 5α-P treated cells in hormone concentrations from 10^{-5} M to 10^{-9} M relative to the control. Data presented as mean ± SEM (t-test, n=5) western blot band optical density.

* significantly different at $p<0.05$ from control.

** significantly different at $p<0.01$ from control.

*** significantly different at $p<0.01$ from control.

Note: 3α-HP data points are slightly offset to avoid overlapping symbols.

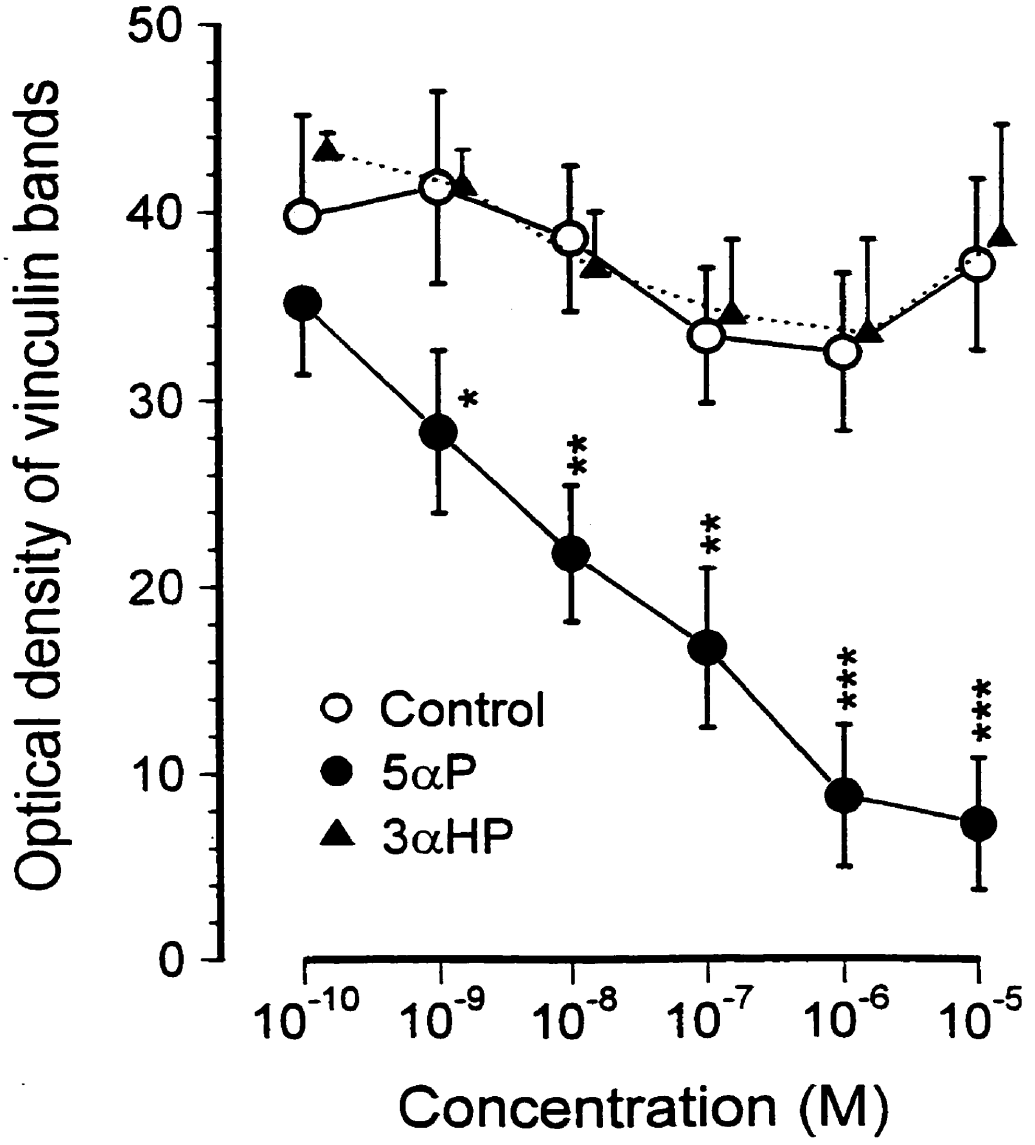

Optical density of vinculin bands
50
40
30
20
10
0
Control
5αP
3αHP
*
**
**
***
***
10-10
10-9
10-8
10-7
10-6
10-5
Concentration (M)

Figure 20: The effects of 5α-P and 3α-HP on the cellular morphology of subconfluent MCF-7 cells in culture. Cells were grown for 3 days with no supplementary hormones (A and B), 10^{-6} M 5α-P (C and D), or 10^{-6} M 3α-HP (E and F). Examination of the photomicrographs reveals that 10^{-6} M 5α-P (C and D) produces noticeable withdrawal of the cellular cytoplasm, leaving behind lamelipodia and filopodia like processes and discontinuous cell-to-cell contact. In many 5α-P treated cells the cellular cross-sectional area is decreased. Treatment with 3α-HP shows no effect on morphology or cellular cross-sectional area. A, C, E 110x. B, D, F 260x.

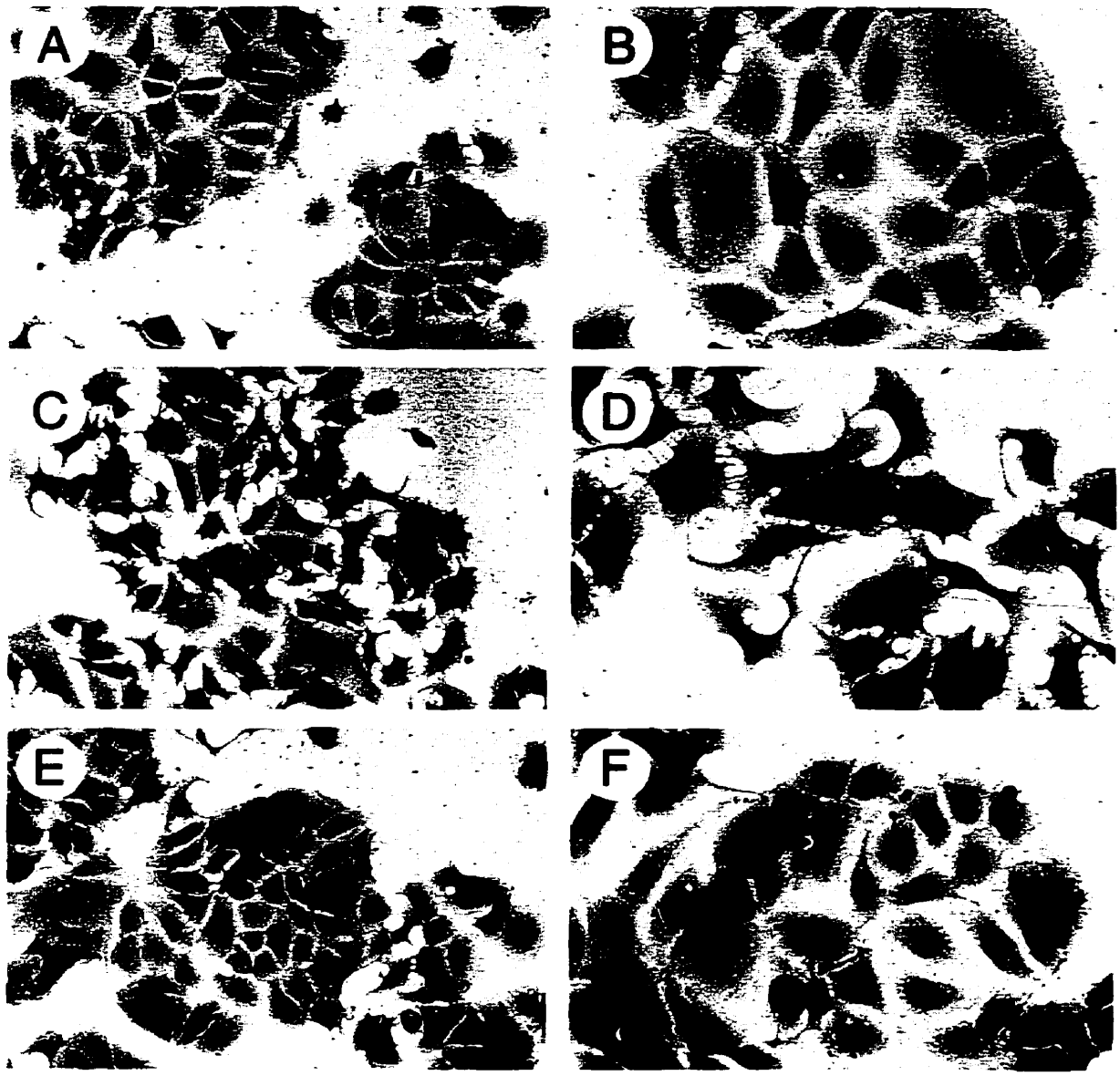

A
B
C
D
E
F

Figure 21: The effects of 5α-P and 3α-HP on the cellular cross sectional area of subconfluent MCF-7 cells in culture. Cellular cross sectional area was quantified by 2-D computer image analysis, as described in *Materials and Methods*. Values are presented as cross-sectional area of individual MCF-7 cells and are mean ± SEM (t-test, n=10 experiments, 150 cells/experiment).

Within each treatment group, bars labeled with different letters are significantly different at $p<0.01$.

* significantly different at $p<0.01$ compared to both control and 3α-HP treatment.

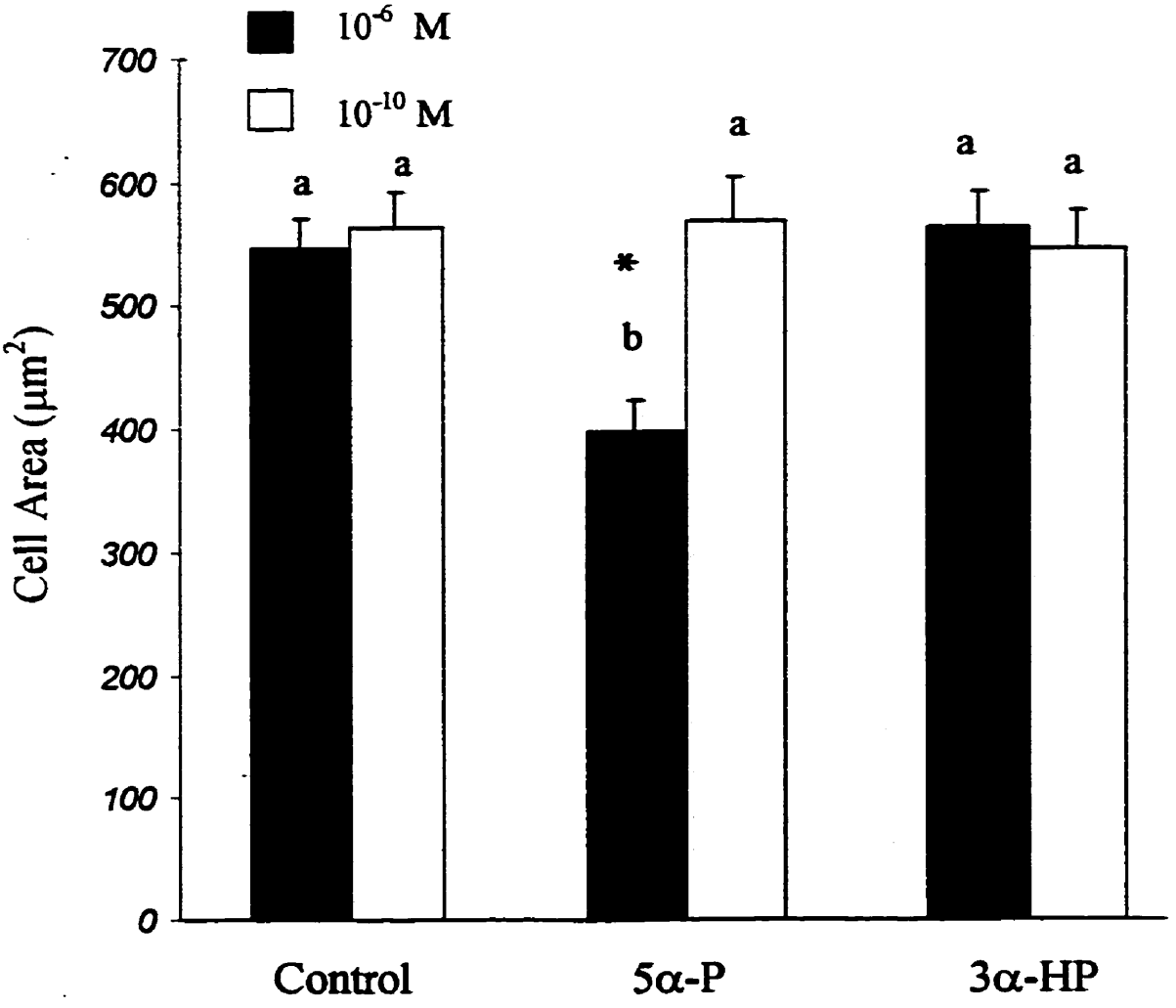

10-6 M
10-10 M
Cell Area (µm2)
700
600
500
400
300
200
100
0
a
a
*
b
a
a
a
a
Control
5α-P
3α-HP

Figure 22: The effects of 5α-P and 3α-HP on the index of cellular shape of subconfluent MCF-7 cells in culture. Index of cellular shape was quantified by 2-D computer image analysis, as described in *Materials and Methods*. Values are presented as index of cellular shape of individual MCF-7 cells and are mean ± SEM (t-test, n=10 experiments, 150 cells/experiment).

Within each treatment group, bars labeled with different letters are significantly different at $p<0.01$.

* significantly different at $p<0.01$ compared to control and 3α-HP treatment.

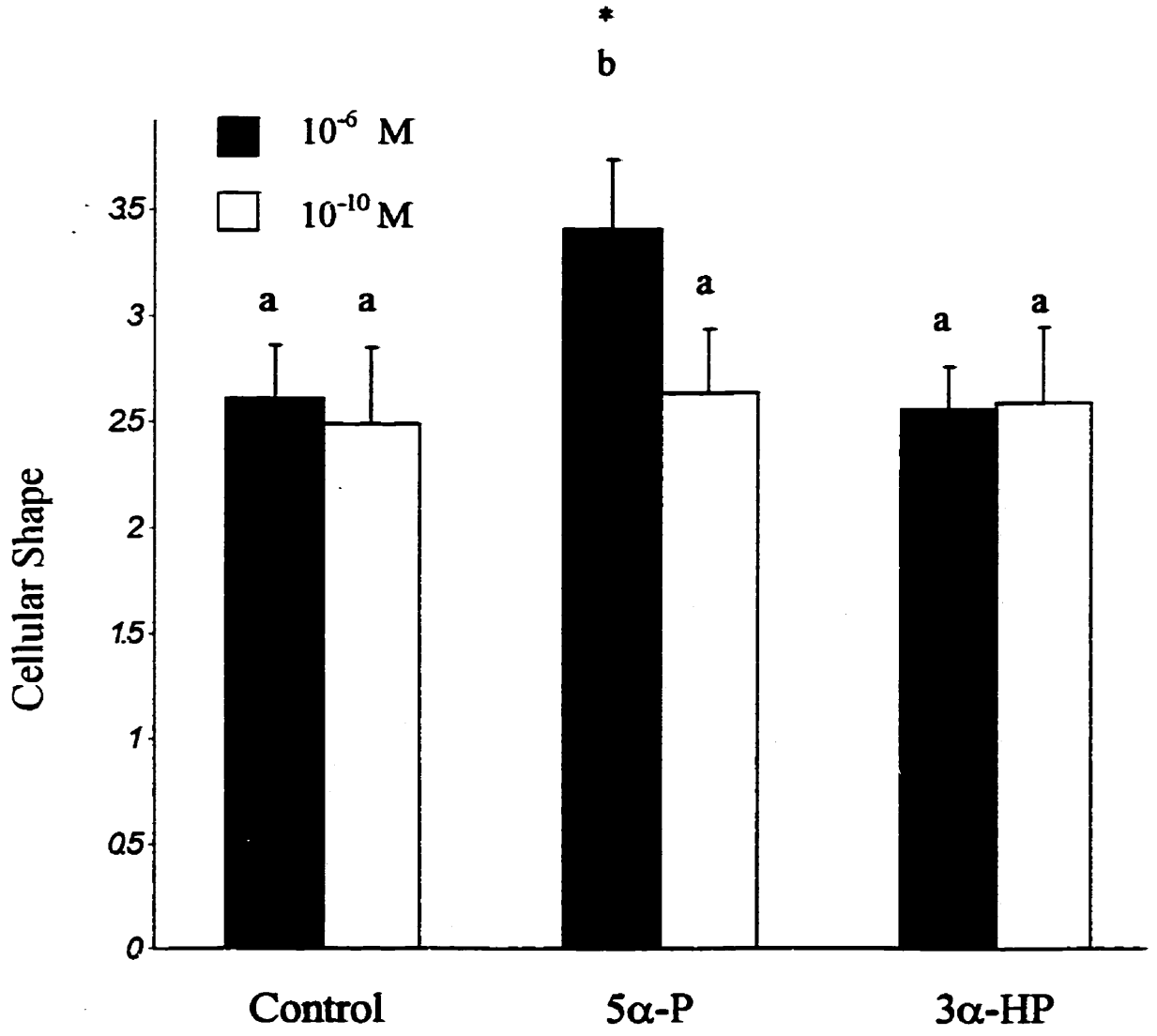
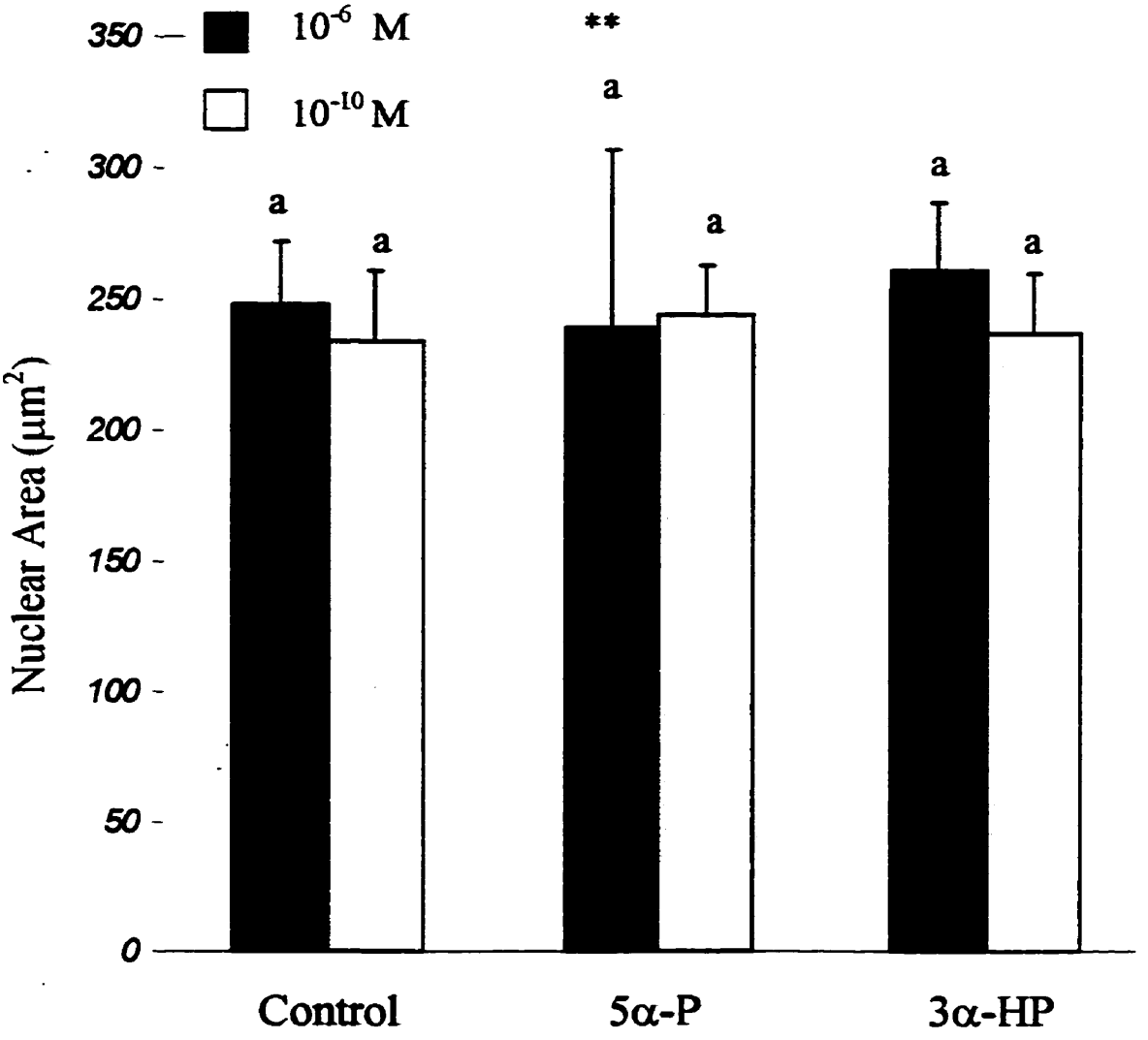

*
b
10^{-6} M
10^{-10} M
a
a
a
a
a
Cellular Shape
3.5
3
2.5
2
1.5
1
0.5
0
Control
5α-P
3α-HP

Figure 23: The effects of 5α-P and 3α-HP on the nuclear cross-sectional area of subconfluent MCF-7 cells in culture. Nuclear cross-sectional area was quantified by computer image analysis, as described in *Materials and Methods*. Values are presented as index of cellular shape of individual MCF-7 cells and are mean ± SEM (t-test, n=10 experiments, 150 cells/experiment).

Within each treatment group, bars labeled with different letters are significantly different at $p<0.01$.

** significantly different variance at $p<0.01$ both within and between treatment groups.


350
300
250
200
150
100
50
0
Nuclear Area (µm²)
10⁻⁶ M
10⁻¹⁰ M
**
a
a
a
a
a
a
Control
5α-P
3α-HP

DISCUSSION

The Effect of 5αP and 3α-HP on Cellular Adhesion

Cellular adhesion is a critical aspect of cancer biology, as invasion and metastasis of tumor cells are the primary causes for the fatal result of malignant cancer (Maemura and Dickson, 1994). Cell metastasis requires that a cell be able to detach from a substrate, travel through tissue and the bloodstream, and attach to different tissue at a another location (Liotta, 1986). This interaction of the cell with its environment is critical for metastasis, as only cells that are able to perform these steps are able to metastasize (Maemura and Dickson, 1994). One of the first steps of this process requires that a change in the adhesion of a cell occur, because if cell adhesion is altered then it is possible for a cell to move and travel throughout the body.

The adhesion of MCF-7 cells to the substrate was significantly decreased by treatment of MCF-7 cells with 5αP. Cells treated with 5αP showed a significant, dose dependant increase in the levels of cellular detachment and a corresponding decrease in the levels of cellular attachment. This agrees with observations that show that cellular adhesion is decreased in cells that have an increase in rate of cellular proliferation and tumorigenicity such as breast (Lewalle *et al.*, 1997), bone (Usson *et al.*, 1997), and lung cells (Takenga *et al.*, 1998). Cells treated with compounds that stimulate growth such as estrogen (Fujimoto *et al.*, 1996), progesterone (Shi *et al.*, 1994; Pearson and Sheldon, 1995), and gluccocorticoids (Gronowicz and McCarthy, 1995) have been shown to display a decrease in adhesion to the substrate and other cells.

Cells treated with 3α-HP showed a significant increase in the levels of cellular detachment and a corresponding increase in the levels of cellular attachment. This agrees with studies that show that the adhesion of cells may be increased in cells that have a decreased rate of proliferation (Ben-Ze'ev, 1985). Osteosarcoma cells treated with 1,25-hydroxyvitamin D_3, a compound that is known to inhibit cell proliferation, display an increase in cellular adhesion (Franceshi *et al.*, 1987). Steroid hormones have also been found to increase the adhesion of platelets to artificial substrates; estrone, progesterone, and 17β-estradiol have been found to increase the adhesion of

these cells by increasing the number of fibrinogen receptors on the cell surface (Chandy and Sharma, 1991). It has been established here that the adhesion of MCF-7 breast cancer cells *in vitro* is decreased by treatment with 5αP and increased by treatment with 3α-HP, confirming the findings of other studies that link rate cellular proliferation with cellular adhesion (Ben-Ze'ev, 1985; Holth *et al.*, 1998).

The Effect of 5αP and 3α-HP on the Distribution of Focal Adhesions and Expression of Vinculin

The effect of transformation on the distribution and expression of the focal adhesion plaque protein vinculin has been studied extensively (Schevzov *et al.*, 1995). Viral transformation of chicken embryonic fibroblasts decreases vinculin expression and organization, and also decreases adhesion of the cell to the substrate (Lee and Otto, 1996). Metastatic cells were found to decrease expression of vinculin and talin relative to normal cells, which also corresponds to a decrease in the size of the cell (Schevzov *et al.*, 1995). Vinculin expression has been shown to be influenced by the state of actin polymerization, with increased polymerization of actin resulting in an increase in vinculin expression (Bershadsky *et al.*, 1995).

Decreases in vinculin expression and organization, and corresponding decreases in number and size of adhesion plaques, were seen in MCF-7 cells treated with 5α-P. The total numbers of focal adhesions, the size of focal adhesions, and the expression of vinculin were reduced in cells treated with 5αP at concentrations between 10^{-6}M and 10^{-8}M. Treatment of MCF-7 cells with 5αP altered the organization of the focal adhesions of MCF-7 cells, with an almost complete loss of focal adhesions within treated cells. These findings agree with previous studies that indicate an increase in cellular proliferation and/or metastatic ability coincides with a decrease in cellular adhesion and organization of adhesion plaques (Sapino *et al.*, 1986; Shi *et al.*, 1994; Suzuki *et al.*, 1998; Wang *et al.*, 1998).

The distribution of focal adhesions and the expression of vinculin was not significantly altered when MCF-7 cells were treated with 3α-HP, although a slight trend towards increasing numbers and size of adhesion plaques was seen in cells treated with 10^{-8}M to 10^{-6}M 3α-HP. This trend agrees with the findings that show

that 3α-HP treated cells are more adhered to the substrate than untreated cells. An increase in adhesion should be seen in an increase in numbers and/or size of adhesion plaques, something that did not occur significantly in 3α-HP treated cells. Possible reasons for this are a) the method of measuring differences in adhesion plaque distribution is not sensitive enough to detect a slight difference between control and treated cells or b) the adhesion plaques of MCF-7 cells may not all use vinculin as a binding protein (Hazan, 1997). Although these plaques share many of the same structural proteins, there are differences between focal adhesions in composition of structural proteins and class of adhesion receptors in the membrane (Hazan *et al.*, 1997).

Cells adhere to the substrate and to other cells via cell-cell and cell-substrate adhesion plaques. Cell-cell focal adhesion plaques contain cadherins (Humphries and Newham, 1998) while cell-substrate adhesion plaques contain integrins (Humphries and Newham, 1998). In MCF-7 cells vinculin is present at the integrin mediated cell-substrate adhesion sites while at the cadherin mediated cell-cell adhesion sites it is α-catenin that binds the end of the actin filament to the cadherin complex (Hazan *et al.*, 1997). α-catenin competitively binds to cadherin complexes, outcompeting vinculin for cadherin binding (Hazan *et al.*, 1997). In the absence of α-catenin, vinculin will bind to the cadherin complex, theoretically assuming the same role. Since MCF-7 cells express both vinculin and α-catenin, it is possible that 3α-HP treatment of MCF-7 cells resulted in an alteration in the number of cell-cell adhesion sites. An increase in the number of cell-cell adhesion sites would increase the adhesion of the cell, while not affecting the numbers or size of vinculin containing adhesion plaques.

The Effect of 5αP and 3α-HP on Distribution of the Actin Cytoskeleton

The distribution of the actin cytoskeleton is critical to a large number of cellular functions including cell growth, motility, and signal transduction. Growth regulation through anchorage dependance (Burridge, 1986), cellular density dependence (Holley, 1975), and cell-shape mediated growth (Farmer and Dike, 1989) are all intrinsically linked to the organization of the actin cytoskeleton. Actin polymerization has been shown to be a key mechanism in signal transduction (Luna and Hitt, 1992), with

extracellular signals such as growth factors and intracellular signals from the ras family of oncogenes being mediated by the actin cytoskeleton (Hall, 1994; Ridley and Hall, 1994).

Due to the many critical cellular functions the actin cytoskeleton performs, alterations in the distribution of the actin cytoskeleton can have dramatic effects on the physiology and behavior of the cell. It is known that cancerous cells possess a cytoskeleton organized differently from normal cells (Runger-Brandle and Gabbiani, 1982; Suzuki *et al.*, 1998), and that highly metastatic cells possess a different cytoskeletal organization when compared to low metastatic cells (Sadano *et al.*, 1992; Suzuki *et al.*, 1998). Changes that occur in the organization and distribution of the cytoskeleton when a cell becomes transformed, or when the ability of the cell to metastasize increases, are highly dependent on the type of cell being studied. The majority of cells, such as endometrial (Koukouritaki *et al.*, 1997), granulosa (Ben-Ze'ev and Amsterdam, 1989), breast (Matsui and Machado-Santelli, 1997), epithelial (Iwig *et al.*, 1995), and salivary (Suzuki *et al.*, 1998) all have decreased levels of cytoskeletal polymerization when the rate of cellular proliferation and metastasis is increased. Some cell types have been shown to express localized increases in actin polymerization within the region of cellular structures such as filopodia (Gabbiani, 1979; Chapponier and Gabbiani, 1989), structures that are necessary for cell motility and metastasis (Sheetz *et al.*, 1998). The present study establishes that the density of the actin cytoskeleton and the amount of actin polymerization is significantly decreased in MCF-7 cells treated with concentrations of 5α-P between 10^{-8}M and 10^{-6} M. This agrees with previous studies that indicate that the majority of cells that have increased rates of proliferation or increased tumorigenicity have decreases in the degree of polymerization of the actin cytoskeleton (Ben-Ze'ev, 1985; Holme, 1990; Berx *et al.*, 1998; Holth *et al.*, 1998).

MCF-7 cells treated with concentrations of 3α-HP between 10^{-8}M and 10^{-6} M, though not significantly different from control cells, show a slight trend towards an increase in numbers of actin stress fibres and the amount of actin polymerization. This slight increase could indicate that cells treated with 3α-HP have decreased tumorigenicity, as cells that have decreased rates of proliferation and metastasis generally show an increase in cytoskeletal organization and polymerization (Ben-Ze'ev 1985; Holth *et al.*, 1998).

This agrees with previous studies that have shown that 3α-HP is found in higher concentrations in non-tumorous tissue than tumorous tissue (Wiebe *et al.*, 1999), and that 3α-HP decreases rate of cellular proliferation in breast cancer cells (Wiebe *et al.*, 1999). It is possible that treatment of MCF-7 cells with 5α-P increases tumorigenicity, thus causing a decrease in actin polymerization, while treatment with 3α-HP decreases tumorigenicity, increasing levels of actin polymerization.

Alterations in organization of the actin cytoskeleton are controlled primarily at the level of polymerization of the actin microfilament and stress fibre, not at the level of actin synthesis (Low *et al.*, 1981; Holme, 1990). This agrees with the present study that indicates actin expression was not altered by treatment with 5α-P, but that actin organization was dramatically altered. Actin exists in equilibrium between the cytoplasmic pool of monomeric (G-actin) and the microfilament structures of polymeric (F-actin) (Schmidt and Hall, 1998). In 5αP treated cells there was a significant increase in amount of G-actin and a corresponding decrease in the amount of F-actin, results that agree with a decrease in stress fibre numbers and organization.

The actin cytoskeleton is made up of actin monomers, microfilaments, stress fibers, and actin bundling proteins (Winsor and Schiebel, 1997). Each monomer is able to bind a molecule of ATP, which is hydrolyzed to ADP after incorporation of the actin molecule into a polymer (Schmidt and Hall, 1998). Polymers assemble spontaneously via non-covalent interactions between the monomeric subunits and are highly dynamic structures with subunit turnover at both ends (Schmidt and Hall, 1998). Microfilaments of actin are able to bind together, with the help of actin-bundling proteins, to form larger structures termed stress fibers (Janmey, 1998). The expression and behavior of actin bundling proteins such as gelsolin (Asch *et al.*, 1996), tropomyosin (Takenga *et al.*, 1988), and actin linking proteins such as α-actinin (Hazan, 1997) and actinin-4 (Honda *et al*, 1998) play a crucial role in the ability of the cell to organize the actin cytoskeleton. Cells treated with 5α-P are not able to form stress fibres possibly because expression or ability of the actin bundling proteins has been decreased, something that is often seen in transformed cells (Takenga *et al.*, 1988; Vandekerhove, 1990; Asch *et al.*, 1996).

The Effect of 5αP and 3α-HP on Cytoplasmic and Nuclear Morphology of MCF-7 Cells

Numerous factors in the cellular environment affect the morphology of cells *in vitro* (Folkman and Moscona, 1978). Among these are cell density (Folkman and Moscona, 1978), growth factors (Zheng *et al.*, 1998), calcium concentration (Constantin *et al.*, 1998), presence/absence of serum in culture media (Medrano *et al.*, 1990), hormonal stimulation (Brown *et al.*, 1998) and growth substrate (Clubb, 1993; Kapur and Rudolph, 1998). Computer assisted morphometry has been used to determine if there are particular downstream cellular events (such as morphology) that can be tied to particular molecular dysfuctions (Giuliano, 1996). It is hoped that morphometry may be useful in dissecting the molecular events occurring within cancer cells (Giuliano, 1996).

An alteration in cellular tumorigenicity is known to dramatically affect cellular morphology (Sapino *et al.*, 1985; Smolle *et al.*, 1992), with well described differences between normal and transformed cells (Suzuki *et al.*, 1998). MCF-7 cells are well described morphologically (Sapino *et al.*, 1985; Osborne *et al.*, 1987), as are their reactions to several different kinds of growth inhibitors and stimulators (Sapino *et al.*, 1986; Gill *et al.*, 1987; Valette, A. *et al.*, 1987). When treated with 10^{-8} M to 10^{-6}M 5αP MCF-7 cells were significantly reduced in area and their cellular shape became much more irregular, something that occurs when steroid hormones increase the rate of cellular proliferation (Sapino, 1986; Darbre and King, 1987). Cellular structures such as filopodia, lameleipodia, and cellular microspikes became increasingly more common, with cell-cell contacts becoming discontinuous. Changes in morphology can indicate, as they have in this study, a possible alteration in cellular adhesion (Raz and Geiber, 1982) and cytoskeletal organization (Goldstein and Leavitt *et al.*, 1985). Although cellular morphometry can be useful in determining structural events within a cell, as it has in this study, the predictive value of morphometry and cancer will not be definitive until more research is done to confirm the link between cell shape and cell function.

The predictive value of nuclear grade of breast cancer has long been known, with alterations in nuclear size and shape commonly being linked to malignant cells (Black

et al., 1997). Before computer assisted analysis, only the largest differences could be seen between normal and cancerous cells (Wolberg *at al.*, 1997). Recent computer cell image analyses have shown that larger nuclear size (Baak *et al.*, 1982; Wittekind and Schulte, 1987) and variation in nuclear size (Stenkvist *et al.*, 1979; Baak *et al.*, 1985) are correlated to poor cancer prognosis. Poor cancer prognosis generally means development of distant metastases, primarily within bone, lung, and axial lymph nodes (Fischer *et al.*, 1968; Baak *et al.*, 1985; Umbricht *et al.*, 1989). Nuclear morphology of "normal" MCF-7 cells is not well described, with studies primarily focusing on the description of different cell clones and on the composition of chromatin within the cells (Komitowski *et al.*, 1994). The nuclear morphology of MCF-7 cells treated with 5αP in concentrations between 10^{-8} M to 10^{-6} M was altered, with an increase in the variability of the nuclear area with increasing concentration of 5αP treatment. Cells treated with 3α-HP did not display a significant alteration in nuclear morphology when compared to control cells. This finding indicates that the tumorigenicity of the MCF-7 cells could be increased due to treatment with 5αP, while 3α-HP does not affect the tumorigenicity of MCF-7 cells.

MCF-7 cells, when stimulated by 5αP, displayed an increase in variation in nuclear area with no change in average nuclear size. These results do not completely agree with previous studies that show that increased metastatic capability is associated with alterations in nuclear size and shape (Baak *et al.*, 1982; Wittekind and Schulte, 1987). A possible explanation for this is that previous studies utilizing morphometry in breast cancer deal exclusively with the comparison of normal cells to cancerous ones (Clark, 1996). This study compares an already immortalized cell line to an immortalized cell line that has been treated with steroid hormones, so many of the large scale morphological alterations may have already occurred. That we see variation in nuclear size being altered by treatment with 5αP without an alteration in average nuclear area shows that although a nuclear morphological alteration is occurring, it is not the same as the difference between normal and transformed cells.

Possible Mechanisms of Action of 5αP and 3α-HP

MCF-7 breast cancer cells treated with 5αP show a generalized disassembly of the actin cytoskeleton within the cell, loss of cellular adhesion, and alterations in cellular morphology. MCF-7 cells possess membrane steroid hormone receptors (Weiler, 1999) to which 5αP and 3α-HP are capable of binding, and it is known that 3α-HP is able to use this membrane bound receptor to stimulate signal transduction (Dhaventari and Wiebe, 1994a; Wiebe, 1997). It is also possible that progesterone metabolites may be able to stimulate the pregnane X receptor, another possible pathway through which 5αP may act (Kliewer *et al.*, 1988). It is known that steroid hormones are able to signal and modulate G-proteins within several different types of cells including Leydig cells (Weiss-Messer *et al.*, 1996), breast cells (Livingston *et al.*, 1998), and brain cells (Caldwell *et al.*, 1997). It is possible that these steroid hormone receptors are able to influence membrane bound G-proteins in order to transduce the signal to the cytoskeleton. G-proteins are known to affect both phosphoinositide levels and the rho superfamily of GTP binding proteins (via tyrosine kinases) activity levels *in vitro*.

The level of phospatidylinositol 4,5-bisphosphate (PIP_2) is regulated by membrane bound G-proteins, and it is possible that PIP_2 or its metabolites are the controlling mechanism behind actin cytoskeleton organization (Janmey and Stossel, 1989). Activation of G-protein activates phospholipase C which cleaves PIP_2 into two different second messengers: diacylglycerol (DAG) and inositol triphosphate (IP_3) (Thomas *et al.*, 1990). The presence of PIP_2 is known to be capable of dissociating the actin bundling proteins profilin and gelsolin from actin (Janmey and Stossel, 1989). Increases in the levels of PIP_2, either through increases in its synthesis or a reduction of rate of metabolism PIP_2 into DAG and IP_3, could result in depolymerization of the actin cytoskeleton (Janmey and Stossel, 1989). It is also known that IP_3 stimulates the release of Ca^{+2} from intracellular stores and that many actin bundling proteins are Ca^{+2} sensitive (Vandekerhove, 1990). Increases in Ca^{+2} could alter the binding capabilities of actin bundling proteins, also causing stress fibers to depolymerize (Vandekerhove, 1990). This agrees with studies that show that the control of calcium levels within cells can be controlled by progesterone

metabolites. The progesterone metabolite 3α-HP is able to alter the concentration of calcium within pituitary cells, an effect that could alter the organization of the cytoskeleton and cellular adhesion (Dhavantari and Wiebe, 1994; Beck *et al.*, 1997). Research has not been performed and it is not known, at this point, whether this regulatory system is responsible for the dramatic changes seen in MCF-7 cells treated with 5αP.

It is well known that G-proteins are able to stimulate tyrosine kinases within cells (Hall, 1994). Tyrosine kinases occur primarily at the terminal actin points where actin fibers interact with points of cell-cell and cell-substrate adhesion (O'Brien *et al.*, 1997). It also well known that tyrosine kinases are able to regulate the assembly of actin and focal adhesions within cells (Kornberg *et al.*, 1992; Burridge *et al.*, 1993). Tyrosine kinases are able to do this by stimulating members of the rho GTP-binding protein superfamily (Egan &Weinberg, 1993). Rho GTPases are key regulators in signaling pathways that link extracellular growth signals or intracellular stimuli to the assembly and organization to he actin cytoskeleton (Van Aelst and D'Souza-Schorey, 1997; Schmidt and Hall, 1998). Members of the Rho family of proteins are activated by specific extracellular signals to direct the organization of the actin cytoskeleton to produce morphological alterations within the cell (Schmidt and Hall, 1998). Rac (a Rho family member) is able to stimulate formation of lameleipodia via stimulation of actin polymerization and alterations in structure of focal adhesions (Nobes & Hall, 1995). Activation of another Rho family member, Cdc42, results in formation of cellular microspikes, filopodia, and an alteration in the organization of cellular adhesion plaques (Kozma *et al.*, 1995; Nobes and Hall, 1995). Rho itself (RhoA) controls the creation of stress fibers and focal adhesions in fibroblasts in response to growth factors and other extracellular signals (Ridley & Hall, 1992). Stress fiber and focal adhesion formation can be blocked by inhibiting the Rho phosphorylation pathway (Paterson *et al.*, 1990). It is possible that the steroid hormones 5α-P and 3α-HP affect alterations in the actin cytoskeleton, focal adhesion plaques, cellular adhesion and cellular morphology by stimulating or inhibiting members of the Rho superfamily of organizational proteins.

Though these aspects of the control of the actin cytoskeleton organization are known, no research has been done as yet that prove a link between the steroid hormones 5αP and 3α-HP and these systems. This study has shown that the steroid hormones 5αP and, to a lesser extent 3α-HP, are able to alter several structural features of MCF-7 breast cancer cells. Cellular adhesion, distribution of the actin cytoskeleton and focal adhesion plaques, expression of the cytoskeletal protein vinculin, and both cytoplasmic and nuclear morphometry are all altered by treatment with 5αP and 3α-HP. At this time the mechanism of action of the progesterone metabolites 5αP and 3α-HP, either directly or indirectly, on such an important system as the cellular anchorage and cytoskeleton is not known.

Summary

1. The cellular adhesion of MCF-7 cells is decreased upon treatment of cells with 5αP *in vitro*, while cellular adhesion of MCF-7 cells is increased upon treatment of cells with 3αHP.

2. The distribution of vinculin containing adhesion plaques is altered in MCF-7 cells treated with 5α-P. Treatment with 5αP results in a significant loss of vinculin containing focal adhesion plaques, while treatment of MCF-7 cells with 3αHP did not result in any significant changes in the distribution of vinculin containing adhesion plaques.

The organization of the actin cytoskeleton is altered in MCF-7 cells treated with 5α-P. Treatment with 5α-P results in an almost complete depolymerization of actin stress fibers throughout the cell. Treatment of MCF-7 cells with 3α-HP did not result in any significant changes in the organization of the actin cytoskeleton.

3. The expression of actin within MCF-7 cells is not significantly altered by treatment with either 5αP or 3α-HP. Treatment of MCF-7 cells with 5αP does increase the concentration of unpolymerized actin and decrease the concentration of

polymerized actin in a dose dependent manner. Treatment with 3α-HP did not significantly affect the concentrations of polymerized or unpolymerized actin within the cell.

Treatment of MCF-7 cells with 5αP in concentrations decreased the intercellular expression of vinculin in a dose dependent manner while treatment of MCF-7 cells with 3α-HP did not significantly affect the intercellular expression of vinculin synthesis within the cell.

4. The cellular area, cellular morphology, and nuclear area of MCF-7 cells were affected by treatment with 5αP. Cellular area was decreased, cell shape complexity was increased, and an increase in variation in nuclear area occurred due to treatment with 5αP. Treatment of MCF-7 cells with 3α-HP did not significantly affect cellular area, cellular morphology, or nuclear area of MCF-7 cells.

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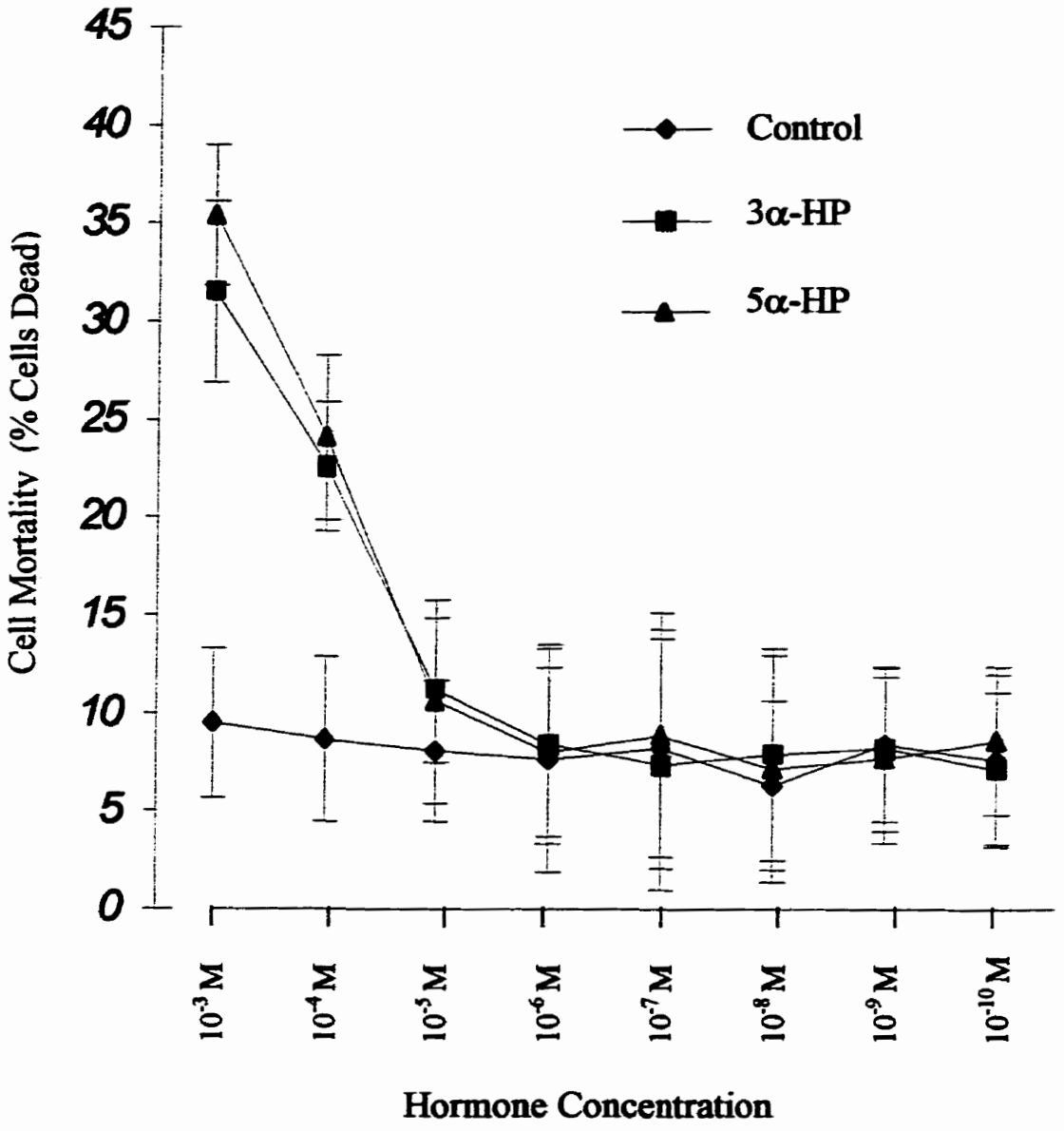
Table 1 Chemicals and their Sources

Sources	Chemicals and Materials
Falcon (Oakville, ONT)	.75 cm^2 culture flasks 25 mm plastic petrie dishes
Sigma (Oakville, ONT)	Dulbecco's Minimal Essential Medium:F12-Ham penicillin streptomycin insulin Trypsin EDTA charcoal dextran T-70 phalloidin-TRITC triton X-100 bovine Serum Albumin mouse monoclonal anti-vinculin antibody mouse monoclonal anti-actin antibody mouse anti-ornithine decarboxylase antibody goat anti-mouse IgG – AP antibody tris-HCL dithiothrietol PMSF sodium dodecyl sulphate imidazole-HCL sodium azide glycerol 5α-pregnane-3,20-dione
Gibco (Missisauga, ONT)	bovine calf serum fungizone
VWR (Missisauga, ONT)	0.2μm syringe filter
Biorad (Toronto, ONT)	nitrocellulose membranes Goat anti-mouse Alkaline Phosphatase Visual Detection Kit

Appendix 2: The effect of 5α-P and 3α-HP on the mortality of MCF-7 cells in culture. Cellular mortality was determined as described in *Materials and Methods*. Values are presented as mean ±SEM (n=3) percentage of MCF-7 cells dead/culture flask. A significant increase in cell mortality occurs when cells are treated with concentrations of 5α-P or 3α-HP are greater than or equal to 10^{-4} M. Cells treated with either hormone at concentrations equal to 10^{-5} M show a slight increase in mortality that is not statistically significant. Cells treated with concentrations of hormones equal to or less 10^{-6} M show no change in cellular mortality.

* both 3α-HP and 5α-P treated cells significantly different from control at $p<0.05$.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40
35
30
25
20
15
10
5
0
Cell Mortality (% Cells Dead)
Control
3α-HP
5α-HP
10-3 M
10-4 M
10-5 M
10-6 M
10-7 M
10-8 M
10-9 M
10-10 M
Hormone Concentration