HORMONE AND GROWTH FACTOR EFFECTS ON THE GROWTH OF HUMAN MAMMARY EPITHELIAL CELLS IN SERUM-FREE PRIMARY CULTURE

by

BRETT MICHAEL GABELMAN

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Department of Anatomy

The University of British Columbia
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THESIS ABSTRACT

The ovarian steroid 17β-estradiol (E_2) is critically involved in the growth control of both normal and malignant mammary epithelial cells (MEC) *in vivo*. However, it has not yet been determined if E_2 directly stimulates the growth of MEC, or if it stimulates growth via production of locally acting autocrine and paracrine growth factors. Epidermal growth factor (EGF) and transforming growth factor-α (TGF-α) are both peptide growth factors which interact with the EGF receptor (EGFR) to stimulate the growth of MEC *in vivo* and *in vitro*. E_2-stimulated growth in human breast cancer cell lines has been shown to be accompanied by increased production of EGF, TGF-α and EGFR. In this thesis the effects of E_2, EGF and TGF-α, alone and in combination, on the growth of human MEC (HMEC) in primary culture were examined. HMEC from reduction mammoplasties, fibroadenomas and carcinomas were cultured on collagen-coated dishes in serum-free medium (DME:F12 (1:1), 5 mg/ml BSA, 10 ng/ml cholera toxin, 0.5 μg/ml cortisol, 10 μg/ml insulin) in the presence and absence of E_2, EGF and TGF-α. Tritiated-thymidine (^{3}H-TdR) incorporation into DNA was used as a measure of cell growth. E_2, at concentrations of 1-1000 nM, did not stimulate growth of any of the cultures examined in the serum-free medium described above. However, E_2 stimulated growth of 1 culture in medium with a reduced insulin concentration (0.1 μg/ml). E_2 inhibited the growth of HMEC in some cultures from all mammary tissue types examined. E_2 effects on HMEC growth were studied in cells grown on fibroblast feeder layers. E_2 still failed to stimulate growth of the cells, but the growth-inhibitory effects of E_2 differed in cells grown on collagen and fibroblasts. EGF, at concentrations of 1-100 ng/ml, consistently stimulated the growth of HMEC from all mammary tissue types examined. The EGF stimulation of growth was reduced by a monoclonal antibody (MAb 528) against the EGF receptor. TGF-α was equally or more effective in stimulating HMEC growth, although its dose response range was different than that of EGF. E_2 plus EGF synergized in the stimulation of HMEC growth in 33% of the samples examined. These studies suggest that E_2 alone under the conditions used cannot directly stimulate the growth of HMEC in primary culture. However, E_2 can exert effects on HMEC growth via modulation of the cells' response to EGF.

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LIST OF ABBREVIATIONS

E_2	17β-estradiol
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ER	Estrogen receptor
^{3}H-TdR	Tritiated thymidine
HMEC	Human mammary epithelial cells
MAb	Monoclonal antibody
MEC	Mammary epithelial cells
TGF-α	Transforming growth factor-alpha

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CHAPTER 1

INTRODUCTION

1. Growth and Differentiation in the Normal Mammary Gland

The mammary gland represents an exciting biological model system in which to study the mechanisms involved in growth and differentiation. Compared to many other organ systems, where the majority of growth occurs during the fetal and early childhood periods of life, the mammary gland undergoes maximal growth and differentiation in the adult. More specifically, with the onset of ovarian hormone production at puberty there is a dramatic increase in mammary gland growth. An even greater surge of growth occurs at pregnancy. Throughout pregnancy the mammary gland continues to grow and differentiate until the onset of lactation. The specific changes in the hormonal milieu accompanying the various stages of mammary gland development control growth and differentiation in a distinct manner. This will be discussed with reference to experimental evidence from animal model systems and cell culture models where applicable.

1a. Anatomy of the Mammary Gland

The mammary gland is defined as a complex tubuloalveolar gland, the function of which is the production and secretion of milk that both protects and nourishes breast-feeding infants. In terms of a functional description, the mammary gland is composed mainly of a connective tissue stroma (90 % resting, 13 % during pregnancy) and an epithelial parenchyma (10 % resting, 87 % during pregnancy) [Russo and Russo, 1987]. The epithelial and stromal components of the mammary gland are separated by a basement membrane, the molecular components of which are produced by both the epithelial and the stromal cells [Kimata et al., 1985]. The specific extracellular matrix composition of the basement membrane plays a critical role in the control of mammary epithelial cell differentiation [Emerman et al., 1977;

Emerman and Pitelka, 1977]. The majority of the nonpregnant adult female mammary gland is composed of fibrous and fatty connective tissue that plays an important structural and supportive role in the gland.

The epithelial component of the mammary gland is responsible for the production and secretion of milk. The human mammary gland consists of 15-20 major ducts called lactiferous ducts, each of which connects with the body surface through individual openings at the nipple. The regions of the mammary gland drained by each of the lactiferous ducts are termed lobes. Prior to exiting at the nipple, each lactiferous duct becomes dilated to form a lactiferous sinus. The sinuses function in the storage of secretory products during lactation. The lactiferous ducts (interlobar) drain several smaller ducts (intralobar) that drain still smaller ducts (interlobular), which end in specialized epithelial buds or "end-buds". It is these terminal end buds which are ultimately responsible for growth and differentiation into both the ducts and the lobuloalveolar units, depending on the hormonal milieu [Bresciani, 1965; 1968]. Lobuloalveolar units are composed of numerous alveoli and the small ductules draining them. These lobuloalveolar regions of the mammary gland, also called lobules, comprise the secretory portion of each mammary gland lobe. The secretory units are composed of two types of epithelial cells. Secretory cells, which are hormonally stimulated to synthesize and secrete milk, line the lumens of the glands. At the basal surface of these cells are specialized contractile cells, myoepithelial cells, which contract under hormonal stimulation to cause milk ejection.

1b. Summary of the Hormonal Control of Mammary Gland Growth and Differentiation

Prior to puberty, the rate of mammary gland growth parallels growth in the other portions of the body. This is referred to as isometric growth. During puberty, under the influence of estrogens produced by the ovaries, both the connective tissue cells and the ductal end-bud cells undergo dramatic increases in growth rates. The active form of estrogen produced by the ovaries is 17β-estradiol (E_2), and this is the estrogen that will be referred to throughout the rest of this thesis. The growth at puberty is greater than the overall changes in body weight and is referred to as allometric growth. The change in mammary gland size and shape with puberty is due to increased proliferation of stromal cells, increased production of extracellular matrix and increased deposition of fat into mammary gland adipocytes. The increased growth of the ductal epithelium contributes little to the change in size and shape of the mammary gland [Borellini and Oka, 1989].

Growth in the epithelial component of the mammary gland during puberty is due to cell division in the end-buds, leading to the elongation of the ductal system [Bresciani, 1965; 1968]. Dichotomous branching also occurs at the level of the end-bud, and it is this branching that is responsible for the tree-like pattern characteristic of the adult mammary ductal network. Growth and differentiation at the level of the end-buds also gives rise to structures known as alveolar buds. These buds will later develop into the functional secretory structures known as the lobuloalveolar units or lobules [Russo and Russo, 1987].

The lack of normal mammary gland development in ovariectomized animals demonstrates the critical requirement of ovarian hormones in this process. Replacement of E_2 in ovariectomized animals results in normal levels of ductal cell growth, whereas both E_2 and progesterone are required for normal development of the alveolar components of the mammary gland [Tucker, 1974]. Variations in the rate of DNA synthesis by mammary gland

epithelial cells are observed during the menstrual cycle. By examining tritiated-thymidine (^{3}H-TdR) incorporation using histoautoradiography, it has been shown that the DNA-labeling index of mammary gland epithelium decreases during the follicular phase of the menstrual cycle when ovarian hormone levels are low [Masters et al., 1977; Meyer, 1977]. Also observed in these studies was an increase in the labeling index of cells during the luteal phase, when E_2 and progesterone reach peak levels. Other researchers characterized the peak of mitotic division, as indicated by the labeling index, to occur at day 25 of the menstrual cycle [Ferguson and Anderson, 1981]. Following this peak at day 25, there is a peak in programmed cell death, or apoptosis, at day 28. However, the level of monthly growth in the mammary gland is in excess of the level of apoptosis [Ferguson and Anderson, 1981]. This results in a net increase in mammary gland development with each ovulatory cycle, until pregnancy or menopause [Vorherr, 1977].

With the onset of pregnancy there is an increase in the levels of both E_2 and progesterone. Although E_2 in combination with either growth hormone or prolactin is sufficient for stimulation of the growth of the ductal epithelium, E_2 plus progesterone, in combination with either growth hormone or prolactin leads to the massive increases of growth seen in the alveolar component of the mammary gland during pregnancy [Topper and Freeman, 1980]. The degree of growth is such that by the end of pregnancy the epithelial tissue, both ductal and alveolar, has largely displaced the surrounding connective tissue. Thus, in contrast to puberty, the changes in mammary gland size during pregnancy are due largely to increases in growth of the epithelial cells.

Accompanying the progression of pregnancy are increases in the level of prolactin, which together with E_2 and progesterone causes increased alveolar epithelial cell growth [Topper and Freeman, 1980]. Another function of prolactin is the induction of differentiation of alveolar end-buds into functional secretory lobuloalveolar units. However, the elevated levels of

progesterone directly opposes the secretory function of prolactin [Salazar and Tobon, 1974]. Specifically, progesterone inhibits prolactin stimulation of both protein (casein) and carbohydrate (lactose) secretion by the alveolar epithelial cells. As a consequence of the inhibition of secretion by progesterone, the secretory alveolar cells undergo significant hypertrophy and become extremely swollen with secretory products in the weeks prior to parturition [Baldwin and Daniels, 1974; Davis and Bauman, 1974].

After parturition there is a sharp drop in the levels of both E_2 and progesterone, whereas the level of prolactin remains elevated. At this point, secretion of milk into the lumen occurs. The initial secretion produced by the lactating mammary gland is referred to as colostrum. It has an elevated level of maternal immunoglobulins, and functions in the transference of passive immunological protection from the mother to the newborn infant [Jenness, 1974]. Colostrum secretion is limited to one week in humans and over a two to three week period of transition the mammary gland secretion is altered to milk, which has lower levels of immunoglobulins but is rich in fats, sugars and proteins [Butler, 1974]. The differentiated, secretory state is maintained as long as the levels of prolactin remain elevated, which is as long as breast feeding continues.

The maintenance of prolactin levels during suckling involves a specific neuroendocrine arc [Grosvenor and Mena, 1974]. Suckling on the nipple activates the nervous system which in turn acts on the hypothalamus to prevent the release of a prolactin-inhibiting factor which therefore permits secretion of prolactin in the anterior pituitary. Ejection of the milk through the nipple also involves interactions between the nervous system and the endocrine system, resulting in oxytocin release from the posterior pituitary stimulating contraction of the myoepithelial cells and forcing ejection of the milk.

Following the termination of breast feeding, the epithelial portion of the mammary gland undergoes a massive regression due to apoptosis and reinfliltration with adipocytes, fibroblasts

and other connective tissue cells [Russo and Russo, 1987]. Numerous macrophages are responsible for the digestion of the degenerate epithelial tissue. These same cycles of growth and differentiation are repeated with each subsequent pregnancy. It is important to note that the level of glandular development in the mammary gland after postlactational regression remains greater than the levels observed in mammary glands of nulliparous women. The potential relevance of this observation is discussed later with regard to the epidemiology of breast cancer.

The exact role of prolactin in the control of mammary gland growth and differentiation is unclear. Growth hormone, also produced in the anterior pituitary, has a 95 % cDNA and a 85 % amino acid sequence identity to prolactin [Martial et al., 1979; Wallis, 1984]. The similar location of potential disulfide bridging cysteine residues further suggests that the proteins have very similar secondary structures [Kohmoto et al., 1984]. Experimentally ovariectomized and hypophysectomized rodents require E_2, progesterone and prolactin in order to undergo normal lobuloalveolar development at pregnancy. However, in some strains of mice the requirement of prolactin can be replaced with growth hormone and normal growth and differentiation of the mammary gland is observed during pregnancy in these mice. This ability of growth hormone to substitute for prolactin is a species-specific effect and varies even between different strains of mice. These findings are difficult to extrapolate in terms of potential roles for these hormones in normal development of the mammary gland [Imagawa et al., 1990].

The circulating levels of prolactin in humans and rodents show cyclical variations during the ovulatory cycle. The peak levels of serum prolactin in humans are observed during the mid-phase and luteal phase of the menstrual cycle [Vonderhaar, 1987b]. E_2 has been shown to stimulate both hypertrophy and hyperplasia of pituitary lactotrophs that produce prolactin [Vonderhaar, 1987b]. As well, E_2 stimulates the production of prolactin in ovariectomized rats, whereas progesterone inhibits this effect [Chen and Meites, 1970]. These two lines of

evidence suggest that the fluctuations in prolactin during the menstrual cycle are likely a direct result of the corresponding fluctuations in E_2 and progesterone levels.

A final major change in the growth and differentiation of the mammary gland occurs at menopause. The cessation of ovulatory cycling and subsequent drop in circulating E_2 and progesterone levels leads to regression of the epithelial component of the mammary gland. The level of regression is such that the remaining epithelial portions of the gland are limited to the large ducts and some of the secondary branches [Vorherr, 1974]. Accompanying the decreases in epithelial content is the increased deposition of fat in adipocytes and an increase in the amount of fibrous connective tissue. This final stage in mammary gland development is of extreme importance from a clinical viewpoint as this is the stage in which the majority of mammary gland neoplasms appear [Leis, 1978].

1c. *In vivo* Evidence for the Role of E_2 in Growth and Differentiation of the Mammary Gland

The readily apparent differences between mammary glands in male and female animals is perhaps the first line of evidence suggesting a role for sex-linked hormones in the control of mammary gland growth and development. Very early work in this field involved the transplantation of ovarian tissue into male rodents and subsequent documentation of the feminization of the male mammary gland [Engle, 1929; Gardner, 1935]. In the 30-40 years following these pioneering studies, the majority of research investigating the regulation of mammary gland growth and differentiation utilized four major strategies. These demonstrated the role of E_2 in the control of ductal mammary epithelial cell growth. These included:

1. Administration of hormones to prepubescent animals to induce growth of the ductal epithelium [Flux, 1954 a&b].

2. Administration of hormones to animals that were endocrinectomized prepubescently in order to determine which hormones could cause normal ductal development [Mumford, 1957; Richardson, 1955; Vonderhaar et al., 1978].

3. Administration of hormones to mature endocrinectomized animals to determine which hormones were involved in maintenance of the ductal cells [Nandi, 1958; Traurig and Morgan, 1964].

4. In animals in condition 3 above, regression of the mammary gland was allowed to occur, and hormonal replacement was investigated [Ferguson 1956].

Ovariectomy of nonpregnant adult mice results in a dramatic decrease of both ductal and alveolar components of the mammary gland. In these same mice, treatment with E_2 alone causes only ductal growth. However, if the mice are treated with both E_2 and progesterone, there is growth of both the ductal and alveolar components of the mouse mammary gland [Bresciani, 1968; 1971]. Ductal growth is generally thought to be largely independent of progesterone [Sakakura and Nishizuka, 1967; Topper and Freeman, 1980]. However, very high doses of progesterone can replace the standard combination of E_2 and progesterone in stimulating the growth of ductal and alveolar portions of the mammary gland in ovariectomized mice [Seyle, 1940; Haslam, 1988a]. More recent studies have shown conclusively the ability of E_2 interaction with estrogen receptors (ER) to stimulate the production of progesterone receptors directly via transcriptional regulation [Haslam and Levely, 1985]. These findings lead to the hypothesis that E_2 may exert its growth effects via modulation of mammary epithelial cells responsiveness to other factors, such as progesterone. This hypothesis is considered further in a later section.

Haslam's group have also investigated the effect of implanting E_2-releasing polymer pellets directly into the mammary glands of ovariectomized mice [Haslam, 1988b]. The result

of this treatment was the stimulation of end-bud cell growth. The details of this important study are discussed further with respect to the mechanism of E_2 action in Sec. 1e. Other groups have also demonstrated the ability of E_2 implants to stimulate ductal growth in animals which have been ovariectomized [Daniel et al., 1987]. The details of these studies are also discussed in Sec. 1e. McManus and Welsch used subcutaneous implants of E_2 to study the effects of E_2 on the growth of human mammary tissue transplanted into nude mice [McManus and Welsch, 1981 & 1984]. Their findings also demonstrated an E_2 stimulation of growth in the ductal epithelium in the human mammary tissue from noncancerous biopsies. In summary, it appears that E_2 functions principally in the stimulation of ductal growth. This may occur by stimulation of cell growth directly, or by alteration of the cells responsiveness to other growth controlling factors such as progesterone or prolactin, as is discussed further in Sec. 1e.

Another interesting observation has been the sharp rise in plasma E_2 levels in mice just prior to parturition [McCormack and Greenwald, 1974; Shaikh, 1971]. This is followed by a short burst of proliferation in the ductal epithelial cells observed immediately after parturition [Brookreson and Turner, 1959; Griffith and Turner, 1961]. This correlation also supports a role for E_2 in the control of ductal cell growth. E_2, as well as progesterone and either prolactin or growth hormone, is also required for proper growth of the lobuloalveolar portions of the mammary gland during pregnancy [Nandi, 1958; Traurig and Morgan, 1964].

In addition to its role in growth control, E_2 may also have a role in differentiation of the mammary gland. Using the C3H/He Crgl mouse strain, Nandi and coworkers demonstrated that ovariectomy and/or hypophysectomy lead to the loss of alveolar structures in pregnant mice. In these same mice, the administration of E_2 and progesterone together was required to maintain the alveolar structures in their normal form [Nandi, 1958; 1959]. When these mice are ovariectomized, hypophysectomized and adrenalectomized, the requirements for lobuloalveolar maintenance are increased to include prolactin, as well as E_2 and progesterone.

E_2 stimulates the production of prolactin receptors in mouse mammary tissue [Sheth et al., 1978]. By increasing the number of prolactin receptors, E_2 may act as a modulator of mammary epithelial cells ability to respond to differentiation signals.

The requirement of E_2 for the maintenance of alveolar structures during pregnancy and lactation may not be common to all animals. In a different strain of mice than used by Nandi and coworkers, animals undergoing ovariectomy during lactation were still able to continue lactating [Griffith and Turner, 1962; Kuramitzu and Loeb, 1921], suggesting that E_2 was not required to maintain the differentiated state. However, another study using a third strain of mice has shown that lactating mammary gland tissue itself may produce sufficient E_2 to maintain lactational activity [Sheth et al., 1978]. It is also possible that sufficient levels of E_2 to maintain lactation may be produced by endocrine organs other than the ovaries, such as the adrenal glands, or by peripheral tissues such as adipocytes. Therefore, it is difficult to conclude by ovariectomy alone that E_2 is not required for lobuloalveolar maintenance *in vivo*. Conflicting findings such as these, all from the same type of animal model, differing only in the strain of animal used, demonstrate the potential difficulties in extrapolating information from animal models directly to the human situation.

1d. *In vitro* Evidence for the Role of E_2 in Growth and Differentiation of the Mammary Gland

Determination of the hormonal requirements of mammary epithelial cell growth has been facilitated greatly by the *in vitro* techniques of organ culture and cell culture. The development of chemically defined media for mammary gland tissue aided our understanding of the control of mammary gland development [Elias, 1957; Ichinose and Nandi, 1964]. These media allowed studies on the hormonal requirements of mammary gland development to "clearly" define the essential hormones involved. The early studies investigated alveolar development

and the hormonal signals involved in regulating both the growth and differentiation of these structures [Banerjee et al., 1973; Ichinose and Nandi, 1966; Wood et al., 1975]. These studies utilized organ culture to study virgin mammary tissue removed from mice that had been "primed" with injections of both E_2 and progesterone. This treatment alone is insufficient to stimulate alveolar differentiation *in vivo*. If the whole mammary glands are then removed and cultured as organ cultures, alveolar differentiation is induced by the addition of prolactin, insulin and a glucocorticoid (hydrocortisone). These results initially seem contradictory to the *in vivo* studies, in which both E_2 and progesterone are required for lobuloalveolar development to occur. However, it is quite likely that in organ cultures, residual hormones from the priming are carried over with the gland to the culture medium. This carry over effect may also include any number of other hormones and growth factors which might also be involved in alveolar development. The results of organ culture studies are further complicated by the finding that organ cultures of unprimed rats undergo DNA synthesis and lobuloalveolar differentiation in response to treatment with only insulin and prolactin. The addition of E_2 further stimulated this response but was not required [Dilley and Nandi, 1967].

The discrepancies between the *in vivo* studies and the *in vitro* studies utilizing organ culture are intriguing. Presently, there is no sufficient explanation as to why mouse and rat mammary tissue, both of which require E_2 for ductal and alveolar mammary epithelial cell growth *in vivo*, differ so dramatically *in vitro* in organ culture with respect to the requirement of *in vivo* pretreatment with E_2 and progesterone. Interpretation of the organ culture studies is further complicated by the inability (at the time of the original research) to determine the target cells and effector cells of the various hormonal treatments. The importance of this is highlighted by the finding of Shymala and coworkers who demonstrated that the dramatic increase in DNA synthesis in the mammary gland tissue of rodents injected with E_2 occurs first in the connective tissue (16 h) and subsequently in the epithelial tissue (24 h) [Shymala and Ferenczy, 1984]. Although the advent of chemically defined media was hoped to eliminate the

complexities of hormonal interactions *in vivo*, many questions are left unanswered by the use of organ culture.

The finding that enzymatic digestion of finely chopped mammary glands with the bacterial collagenase enzyme produced by *Clostridium histolyticum* resulted in viable fragments of the mammary epithelial tree was a major step in the development of primary cultures of MEC [Lasfargues, 1957]. Lasfargues showed that the epithelial fragments from collagenase digestion were relatively free of fat and stromal cells, which allowed enriched populations of epithelial cells to be examined *in vitro*. Several investigators have studied the effects of E_2 and other mammogenic hormones on the growth of primary cultures of rodent MEC grown on plastic in serum-containing medium. E_2 has either no or a very slight stimulatory effect under these conditions [Ceriani and Blank, 1977; Hallowes et al., 1977; Richards and Nandi, 1978].

The data from studies utilizing primary cultures of rodent MEC grown on biological substrates may provide more relevant information on growth effects of E_2 and other mammogenic hormones, given that cells grown on these substrates are capable of undergoing extensive cytodifferentiation in response to hormones [Emerman and Pitelka, 1977; Emerman et al., 1977]. Studies examining the effects of E_2 on the growth of both mouse and rat MEC have shown that E_2 is unable to stimulate the growth of these cells when grown upon collagen-coated dishes [Edery et al., 1984; Imagawa et al., 1985]. In contrast to these studies, Ethier and coworkers have demonstrated an E_2 stimulation of rat MEC growth on cells grown on collagen-coated dishes in serum-free medium [Ethier, 1986; Ethier et al., 1987]. It is important to note, however, that the serum-free medium used by Ethier's group contained epidermal growth factor (EGF), whereas the serum-free medium used in the studies showing no E_2 effect did not contain EGF. The importance of this is discussed in detail later.

Interpretation of the large body of literature on E_2 effects on the growth and/or differentiation of mammary epithelial cells in primary cell culture is made difficult by the

variation in experimental conditions used by different investigators. The results of the relatively few studies investigating the effects of E_2 on the growth of human MEC (HMEC) have been at least as mixed as those done with rodent cells. E_2 in primary culture can stimulate the proliferation of normal HMEC grown on plastic in the presence of human serum, and it appears that in this same culture system progesterone stimulates differentiation [Mauvais-Jarvis et al., 1986]. Also in normal HMEC in primary culture, E_2 has been shown to stimulate DNA synthesis, possibly due to shortening of the cell cycle length [Calaf et al., 1986]. A common feature of these studies and others demonstrating E_2 stimulation of growth is the presence of serum in the medium [van Bogaert et al., 1982; Longman and Buehring, 1987]. Other effects of E_2 on cultures of mammary epithelial cells include increases in the number of microvilli [Chambon et al., 1984], increased casein production and lactose synthetase activity [Sankaran et al., 1984], and increased tyrosyl-kinase activity [Sheffield et al., 1987]. There are few results of the effects of E_2 on the growth of HMEC in serum-free medium. Yang et al. [1980, 1982] have examined the effect of E_2 on the growth of normal and fibroadenoma HMEC. E_2 has no growth effects in their system in either the presence or absence of serum.

One common criticism of organ culture and cell culture as tools for studying the control of growth and differentiation of the mammary gland has been that investigators are simply studying novel growth requirements for mammary tissues in abnormal conditions. Although this may be partially true, information regarding the direct effects of hormones and interactions among them on growth and differentiation of mammary tissue is still greatly lacking. The technique of cell culture, both of epithelial and stromal cells alone and in combination, provides a tool to more completely understand the actions and mechanisms of action of hormones such as E_2 in the regulation of mammary gland development.

1e. Mechanism of E_2 Action

In blood serum, E_2 is present bound to steroid binding protein. In this form, E_2 cannot readily enter target cells. However, in its free unbound form, E_2 readily migrates through lipid cell membranes due to its cholesterol based structure. Once inside the cell, it interacts with a specific intracellular receptor, the estrogen receptor (ER). The nature of the ER and its intracellular location is still a topic of some debate. The debate centers on whether the ER in its free form resides exclusively inside the nucleus or in both the cytoplasmic and nuclear compartments.

If ER is found in the cytoplasm, then immediately upon binding to E_2, the E_2-ER complex is translocated to the nucleus. Clearly if ER is normally present in the nucleus then translocation is not a relevant step following hormone binding. In either case, the E_2-ER complex has a high affinity for specific regions of DNA. These regions have been termed hormone responsive elements (HRE)[Darbre, 1990]. Upon binding to its specific HRE, the E_2-ER complex functions as a transcriptional regulator. The end result is transcriptional activation of genes involved in the control of target cell growth and differentiation. Specific examples of genes directly effected include *c-myc*, *c-fos*, EGF, TGF-α, and many others [van der Burg et al., 1989, Lippman and Dickson, 1989]. The particular importance of some of these specific transcriptional activations is discussed further in a later section.

The finding that E_2 is generally unable to stimulate the growth of mammary epithelial cells in serum-free primary culture has led to considerable speculation of how E_2 actually functions *in vivo* and in organ culture to stimulate the growth of ductal epithelial cells. Three major hypotheses have been proposed to account for the effects of E_2:

1. E_2 directly stimulates the proliferation of target cells in which it is bound [Aitken and Lippman, 1982].

2. E_2 indirectly stimulates growth by the production of systemically acting factors produced at sites distal to the mammary gland [Sirbasku et al., 1985].

3. E_2 acts both by affecting target cells responsiveness to local autocrine and paracrine growth factors, and by affecting the production of these locally acting growth factors [Lippman et al., 1988].

Both the first and third hypotheses of stimulation of HMEC growth are supported by the finding that E_2 implants into the mammary glands of 5 week old ovariectomized mice stimulates the growth of only the mammary gland into which the E_2 implant is placed. In this case E_2 is not exerting its growth regulatory effects via the production of systemic factors [Haslam, 1988b]. However, if these same experiments are performed on 10 week old mice, the effect of the implant is stimulation of epithelial cell growth in both the implant-receiving mammary gland and the contralateral control mammary gland. In this case E_2 is exerting growth regulatory effects via the induction of a systemic factor. The lack of direct E_2 stimulation of HMEC growth *in vitro* is therefore likely due to the absence of required factors present *in vivo*. These investigators further demonstrated that the ability of E_2 to increase functional levels of progesterone receptors is specific to the tissue from ten week old mice, and does not occur in tissue from 5 week old mice [Haslam, 1988b]. These findings suggest that in this model system E_2 can stimulate growth both directly and systemically, depending on the age of the animal. E_2 only stimulates the production of progesterone receptors at an age when systemic effects are also occurring.

In an elegant series of experiments, Daniel et al. showed that the implantation of E_2-releasing pellets causes increased growth in a specific subset of the end-bud region MEC [Williams and Daniel, 1983] called the cap cells [Daniel et al., 1987]. However, the hormone-binding studies using radiolabelled hormones and autoradiography demonstrated that the actual hormone-binding cells are the stromal cells and another epithelial cell subset of the end

bud region, called the end bud cells, but not the cap cells. The work of Daniel's group also supports the model of E_2 action whereby E_2 exerts its growth regulatory effects via the production of paracrine acting growth factors.

The ability of E_2 to stimulate progesterone receptors in MEC supports the model for E_2 action in which E_2 modulates the responsiveness of the MEC to other hormones and growth factors. E_2 has also been shown to regulate the level of EGF receptors (EGFR) in uterine tissue [Gardner et al., 1989]. The regulation of EGFR by E_2 is discussed further in Sec. 1f.

1f. Role of Epidermal Growth Factor (EGF) and Transforming Growth Factor-α (TGF-α) in Control of Mammary Gland Growth and Differentiation

EGF is a small single chain polypetide consisting of 53 amino acids [Carpenter and Cohen, 1979]. Both EGF and the closely related TGF-α exert their biological effects via interaction with the same transmembrane receptor, the EGFR [Todaro et al., 1980, Massague, 1983, Carpenter, 1987]. Interaction with the receptor activates the receptor kinase portion of the receptor and an intracellular increase in tyrosine phosphorylation is observed following EGF binding. Other cellular responses to EGF include the EGFR activation of phospholipase-C and the subsequent increased production of inositol 1,4,5-triphosphate (IP3) [Schlessinger, 1988]. The immediate substrates of the EGFR tyrosine kinase have not yet been conclusively identified. However, both the progesterone receptor and HER2-neu receptor are rapidly tyrosine phosphorylated in response to EGF treatment [Ghosh-Dastidar et al., 1984; King et al., 1988]. The HER2-neu receptor is another transmembranous growth factor receptor with a tyrosine kinase intracellular domain. HER2-neu is highly homologous to EGFR in its amino acid and DNA sequence, which in the case of HER2-neu is encoded on the c-erbB-2 cellular oncogene [Coussens et al., 1985]. This gene and its encoded receptor will be discussed further with reference to its role in breast cancer in Sec. 2.

It is interesting to note that the EGFR gene belongs to a relatively large family of genes encoding growth factor receptors, many members of which have already been characterized for their potential to undergo oncogenic mutation or activation [Hunter and Cooper, 1985; Yarden and Schlessinger, 1988]. Although no directly oncogenic mutations in the EGFR gene have yet been discovered in breast cancer, amplification of the closely related c-erbB-2 gene and overexpression of its protein product are associated with advanced forms of breast cancer [Slamon et al., 1987]. The importance of EGFR as it applies to breast cancer is discussed later.

Both direct and indirect evidence links EGF to regulation of normal mammary epithelial cell proliferation. High levels of EGF are found in both milk and breast cyst fluid [Zwiebel et al., 1986; Connolly and Rose, 1988]. TGF-α activity has been isolated from normal mammary tissue [Valverius et al., 1987]. Unfortunately it is presently unknown whether normal mammary gland epithelial cells directly produce EGF or TGF-α, or if they sequester the high levels from the surrounding extracellular fluids. The demonstration of mRNA for both EGF and TGF-α in normal mammary tissue from both rodent and human sources suggests that the epithelial cells are likely to produce at least a portion of the EGF and TGF-α isolated from mammary tissue [Brown et al., 1989; Liscia et al., 1990].

The presence of specific high and low affinity receptors on normal MEC further supports a potential role for EGF in the control of growth and/or differentiation [Taketani and Oka, 1983]. EGFR levels vary according to the physiological state of the of the mammary gland. There are significant numbers of EGFR present on the MEC in virgin and lactating glands, which increase to peak levels during pregnancy [Edery et al., 1985]. It is therefore possible that the hormonal factors modulating growth and differentiation during pregnancy also modulate EGFR levels. If these changes in EGFR subsequently increase the cells responsiveness to EGF, then it is conceivable that these effects may be involved in the stimulation of growth and differentiation

observed during pregnancy. This concept is not entirely speculative given that increased production of both EGF and EGFR accompanies estrogenic stimulation of epithelial cell growth in the mouse uterus [Gardner et al., 1989; Huet-Hudson et al., 1990].

Levels of circulating EGF produced by the submandibular gland in mice are regulated by progesterone [Bullock et al., 1975], and progesterone stimulation of growth in breast cancer cell lines is accompanied by increased expression of EGFR [Musgrove et al., 1991]. As mentioned earlier, progesterone is critical to the development of lobuloalveolar structures during pregnancy [Haslam, 1987]. It is therefore possible that progesterone activity is also partially mediated by stimulation of EGF production. Given that E_2 is directly able to regulate progesterone receptor levels (Sec. 1e.), the regulation of progesterone levels in the submandibular gland may function as an important control pathway for the regulation of MEC growth by distal organs, as proposed in the estromedin hypothesis for E_2 action [Sirbasku, 1978].

Direct support for the role of EGF in mammary gland development *in vivo* comes from studies where the level of EGF is experimentally modulated. Pregestational removal of the submandibular gland, the principal source of EGF in mice, results in a dramatic decrease in both mammary gland size and the volume of milk produced by subsequently impregnated mice [Okamoto and Oka, 1984; Sheffield and Welsch, 1987]. When replacement of EGF is provided by injection during pregnancy, both mammary gland size and milk volume return to normal levels [Oka et al., 1988]. However, it was noted that the level of lobuloalveolar development does not return to normal by supplementation with EGF alone. This leads to the hypothesis that other growth regulatory factors are also being produced by the submandibular gland, which might be tentatively labeled as potential estromedins.

Other investigators have used polymer implants similar to those in studies examining E_2 function to analyze the effect of EGF on mammary gland growth and differentiation *in vivo*.

Coleman et al. [1988] showed that implantation of EGF pellets in the mammary glands of ovariectomized mice leads to increased end bud formation. Coleman and co-workers also demonstrated that EGF binding occurred in end-bud cells, ductal myoepithelial cells and in the stromal cells surrounding the responsive epithelial cells. More recently the same group showed that implantation of EGF into intact animals leads to a time-dependent inhibition of growth in the ductal epithelium [Coleman, 1990]. This effect is observed after an exposure to the pellets of at least 3 days. A down regulation of EGFR levels in response to the implants is also observed. The authors suggest that the growth inhibitory effect is likely due to this down regulation of EGFR by the implanted EGF.

In vivo studies with implants have also suggested a possible synergistic effect between EGF and the ovarian steroids E_2 and progesterone [Vonderhaar, 1987]. Vonderhaar examined the effects of implants of both EGF and TGF-α on ductal branching and end-bud formation and growth in ovariectomized mice. EGF stimulates both ductal branching and end-bud formation if both E_2 and progesterone are added in conjunction with the EGF implant. In contrast to EGF, TGF-α stimulates both branching and growth independently of additional hormonal supplementation [Vonderhaar, 1987]. This study suggests that not only may E_2 and/or progesterone be required for the EGF effect, but that the closely related growth factors EGF and TGF-α may have very different biological responses despite interaction with a common receptor pathway.

Tissue culture experiments have demonstrated the ability of EGF to stimulate the growth of breast cancer cell lines [Lippman and Dickson, 1989], and normal MEC from numerous animal models [Yang et al., 1980 & 1986; Salomon et al., 1981; McGrath et al., 1985; Ethier et al., 1990]. In mouse organ culture, Tonelli and coworkers [1980] were able to stimulate a cycle of growth, differentiation and regression by addition of the insulin, prolactin, aldosterone and hydrocortisone to the medium in cultures of mammary glands from virgin mice that have been

primed with E_2 and progesterone injections. However, when EGF was added to the medium in combination with insulin, prolactin, aldosterone and hydrocortisone, the investigators were able to stimulate a second round of development, differentiation and regression, which could not be done without the presence of EGF. The effects of EGF added to primary cell cultures of normal and benign MEC are varied in terms of the growth response. EGF addition to serum-free media has been shown to stimulate growth in cultures of normal and malignant MEC from intact postpubertal nonpregnant mice [Imagawa et al., 1982], ovariectomized and ovariectomized + adrenalectomized mice [Levay-Young et al., 1990] and intact mature nonpregnant rats [McGrath et al., 1985; Ethier, 1985]. Studies by Ethier's group demonstrated a requirement for EGF in serum-free medium for either progesterone, prolactin or E_2 to exert growth modulating effects on cultures of normal rat mammary epithelial cells in monolayer cultures. The growth effects of progesterone and prolactin were reported to vary in individual experiments, although E_2 was reported to cause a slight but consistent stimulation of growth of rat mammary epithelial cells in medium containing EGF [Ethier, 1986; Ethier et al., 1987].

The ability of EGF to stimulate the growth of rat mammary epithelial cells in culture is also dependent on the nature of the substrate upon which the cells are grown. Although EGF stimulates the growth of cells in serum-free medium on either a plastic or Type I collagen substrate, it has no effect on the growth of the same cells on Type IV collagen [Salomon et al., 1981; Kidwell and Shaffer, 1984]. The investigators hypothesize that EGF stimulation of growth on plastic or Type I collagen is due to the stimulation of Type IV collagen production by the cells in response to EGF treatment. The Type IV collagen (basement membrane collagen) likely provides a better substrate for epithelial cell growth than either plastic or Type I collagen and stimulates growth on its own. More recent studies have shown that TGF-α also stimulates the production of Type IV collagen [Liu et al., 1987]. Therefore, the above model explaining EGF stimulation of growth may also be applied to the growth-stimulatory effects of TGF-α on mammary epithelial cells in culture. The importance of substrate and the effects of hormones

and growth factors on both the production and degradation of substrate components is discussed in further detail in the Discussion section of this thesis.

In contrast to the numerous experiments showing stimulation of MEC growth in response to EGF, Ehmann and coworkers reported a growth inhibitory effect of EGF on mouse MEC growth [Ehmann et al., 1984] in serum-containing media. The epithelial cells were grown on a feeder layer of irradiated rat mammary tumor cells. The possible production of unidentified growth factors by the feeder layer or the presence of other growth factors in the serum were not accounted for and make the growth inhibition by EGF difficult to interpret.

Only one other report has shown an absence of growth stimulation by EGF in primary cultures of HMEC. Yang et al. [1986] utilized the same serum-free medium as was used for the experiments described in this thesis and showed that EGF stimulated cells from fibroadenomas only if the cells were grown in three-dimensional cultures. In the two-dimensional culture system used in this thesis, Yang and coworkers found no growth effect with EGF. Potential explanations for the discrepancy in results from Yang's group and those presented here are presented in the Discussion section.

2. Involvement of E_2 and Epidermal Growth Factor in Breast Cancer

Studies of hormones and growth factors in the genesis and progression of breast cancer have examined the involvement of all the hormones and growth factors already discussed with regard to growth control of the normal mammary gland. The literature reviewed here is limited to the roles of E_2 and EGF in the process of breast cancer.

2a. Estrogens and Breast Cancer

Animal studies investigating the role of estrogens in breast cancer have shown that prolonged exposure to estrogens can lead to the induction of mammary tumors [Dunning et al.,

1947; Cutts and Noble, 1964]. Cutts and Noble showed that the incidence of estrogen-induced tumors varies dramatically between different species of rats. The mammary tumors in these animals undergo partial remission in response to ovariectomy or adrenalectomy and total remission in response to hypophysectomy. Therefore, estrogen's role in the control of growth in these tumors is neither complete nor direct, as removal of endogenous estrogens only caused partial regression of tumors, whereas hypophysectomy resulted in total regression of the tumors. These observation have led to debate as to how estrogens might function in the genesis and growth control of breast cancer.

Further animal studies have suggested E_2 likely acts as a permissive or promoting agent rather than a carcinogen. When 3-methylcholanthrene (3-MC), a polycyclic aromatic hydrocarbon that is highly carcinogenic, is administered to female Sprague-Dawley rats in a single feeding results in an incidence of mammary tumors up to 100%, depending on the age of the rats at the time of 3-MC exposure [Huggins, 1961]. In male rats, no mammary tumors are caused by the same treatment, suggesting that female sex hormones play a key role as permissive agents in mammary tumor induction. Ovariectomy prior to 3-MC administration also eliminated the induction of mammary tumors by 3-MC. If ovarian grafts are performed on the same day as carcinogen exposure, there is a partial restoration in the incidence of mammary tumors [Dao, 1962]. However, if ovarian grafting is performed subsequent to carcinogen exposure, the incidence of mammary tumors is unaffected, demonstrating that ovarian hormones are required as permissive agents for the carcinogenic effect.

Animal studies examining the role of ovarian hormones in growth control of established mammary tumors have also utilized carcinogen-induced tumors to a great extent. These studies have shown that ovariectomy leads to temporary regression of mammary tumors [Huggins et al., 1961; Dao, 1962; Gullino et al., 1975]. Although these studies suggest a role for E_2 in the initiation and growth control of breast cancer, they are far from conclusive.

Ovariectomy does reduce circulating E_2 levels, however, it also reduces progesterone levels and possibly levels of other unidentified factors as well. Furthermore, the regression of tumors is only temporary in response to ovariectomy, further demonstrating the importance of other factors in the growth control of mammary tumors. It is possible that mammary tumors are initially dependent on ovarian hormones for growth control and the progression to a hormone-independent state accounts for the temporary regression resulting from ovariectomy. This critical hypothesis is discussed further in consideration of growth factors in breast cancer.

Treatment of numerous ER^+ breast cancer cell lines with E_2, both *in vitro* and in nude mice, leads to an increase in growth in the tumor cells [Lippman et al., 1976; Soule and McGrath, 1980; Darbre et al., 1983; Lippman and Dickson, 1989]. Accompanying the increases in growth are increases in the production of numerous peptide growth factors and their receptors by the cancer cells [Dickson et al., 1986]. The list of such growth factors includes, but is not limited to, EGF and the closely-related TGF-α, insulin-like growth factor-1 (IGF-1), platelet-derived growth factor (PDGF), and basic fibroblast growth factor (bFGF). Growth inhibition of ER^+ cell lines by the antiestrogen tamoxifen is accompanied by a decrease in the level of production of these growth factors, as well as an increase in the production of growth inhibitory substances in some cell lines. In ER^- breast cancer cell lines, which grow at a higher basal rate and are not stimulated by the addition of E_2, these same growth factors are produced autonomously at elevated levels [Davidson and Lippman, 1989].

These observations in cell lines also support a model of mammary tumor progression by the deregulation of the production of autocrine and paracrine growth factors. According to this model, an initial event leads to abnormal production of growth factors in response to E_2. At this stage the tumor is still hormone-dependent, requiring E_2 for continued growth. The animal studies and epidemiological data showing temporary remission of mammary tumors in response to ovariectomy provide strong supportive evidence for this portion of the model. A

second defect in the growth control of these hormone-dependent tumors then results in autonomous production of the same growth factors that were initially under E_2 control. This stage would be the hormone-independent tumors characterized by higher basal growth rates and independence of E_2 for growth. The next section (Sec. 2b.) will consider further evidence supporting an important role of EGF and its receptor in this mechanism of disease progression.

Considerable epidemiological evidence exists linking female sex steroids to a role in the onset and progression of malignant breast disease. Women with early menarche and late menopause appear to have a greater risk of developing breast cancer [Zumoff et al., 1975; Kelsey, 1970], whereas an early onset of menopause or ovariectomy correlates with a reduced risk of breast cancer [Trichopoulos et al., 1972]. This epidemiological evidence is the basis of the estrogen window hypothesis which states that the longer a woman's exposure to E_2, the greater the risk of breast cancer. The total duration of E_2 exposure is defined as the time from puberty to menopause. It has been suggested that it is the exposure to unopposed E_2, that is low progesterone levels, that might increase the risk of breast cancer [Korenman, 1980]. Low levels of progesterone are characteristic of the follicular phase of the menstrual cycle, or they may occur due to deficiencies in normal production of progesterone in the luteal phase. However, this theory is largely speculative and investigators have been unable to find any association between anovulatory cycles (low progesterone) and women with a high incidence of breast cancer [MacMahon et al., 1980]. Proponents of this theory of unopposed estrogen suggest that the reduction in breast cancer incidence in women with a first pregnancy at a young age is due to a protective function of the high doses of progesterone produced during pregnancy [Davidson and Lippman, 1989].

The incidence of breast cancer is significantly increased with obesity and may be due to the fact that fat cells are the principal site for the conversion of androstendione to estrone, the precursor to the family of biologically active estrogens [Davidson and Lippman, 1989].

However, it has not been shown that circulating levels of E_2 are different in obese women than average body weight controls. Also, the levels of E_2 in breast tissue are significantly higher than in serum [James et al., 1971; Witliff, 1974]. The higher levels of adipocytes (which produce E_2 from inactive precursors) present in the breasts of obese women could lead to increased local levels of E_2 in the breast.

Due to the possible role of E_2 in the initiation of breast cancer, numerous studies have been conducted examining the risk of breast cancer in women using hormonal contraceptives containing E_2 and in women utilizing E_2 replacement therapy post-menopausally [Drill, 1981; Thomas, 1982]. The findings in this large body of studies have been varied with respect to increased risk of breast cancer and further studies are required to determine the roles of duration of exposure and dosage of the exogenous E_2. To date there is no convincing evidence that E_2 used medically has any role in the initiation of breast cancer, however, the potential for such a risk warrants further study in this area. In summary then, the experimental and epidemiological evidence suggests a role for estrogens in the breast cancer process.

The strongest evidence for the importance of E_2 as the specific female sex steroid involved in growth control of mammary tumors comes from epidemiological studies on the ER status of breast cancer patients. Approximately 66% of human breast cancers are classified as ER^+ [Clarke et al., 1990]. In this ER^+ group of patients, remission is observed in 70% of cases treated with either E_2 removal via ovariectomy, high dose E_2 therapy or treatment with the antiestrogen tamoxifen. Due to the severity of side effects with E_2 removal or high dose treatment, the current treatment is the use of the relatively low side-effect inducing antiestrogen tamoxifen. These same endocrine treatments yield remission in only 5-10% of ER^- breast cancers. This difference in response represents a progression of the tumors from hormonally-responsive ER^+ cancers to hormonally-independent ER^- tumors [King, 1989]. Progression of the tumors to a hormonally-independent state could occur by a number of

defects in the cell. Unregulated and elevated expression of autocrine and paracrine growth factors which are normally under endocrine control could be a mechanism by which breast cancer cells progress to hormonal independence. Another possibility is that breast cancer cells have an exaggerated response to the autocrine and paracrine growth factors that are produced in response to endocrine hormones. Exaggerated responses to hormonally-regulated growth factors could occur with amplified receptor levels.

2b. EGF and TGF-α in Breast Cancer

The roles of EGF and TGF-α in the growth control of normal mammary tissue has already been discussed and includes a description of both the growth factors and their receptor, EGFR. Experimental animal evidence links EGF to the development of malignant breast disease in mice with a high incidence of breast cancer. If the mice undergo sialoadenectomy (removal of the submandibular gland) prior to 30 weeks of age, the incidence of breast cancer in these mice drops from 63% to 13% at 52 weeks of age [Kurachi et al., 1985]. The importance of the 30 week age limit is that at this time the production of EGF by the submandibular glands in mice is greatly increased and the incidence of mammary tumors rises dramatically following this event. Removal of the submandibular glands after the appearance of mammary tumors in these mice results in a regression of the tumors. If the mice receive EGF injections after sialoadenectomy, the incidence of new and growth of established tumors is returned to elevated levels. These findings demonstrate both promotional and growth-regulatory roles for EGF in mammary carcinogenesis.

Considerable evidence from breast cancer cell lines links both EGF and TGF-α to regulatory roles in breast cancer cell growth. EGF and TGF-α are both produced by breast cancer cell lines [Salomon et al., 1984; Dickson et al., 1985 and 1986] and numerous studies shown that both EGF and TGF-α stimulate the growth of the same cell lines [Lippman and

Dickson, 1989]. Furthermore, E_2 stimulation of ER^+ breast cancer cell line growth is accompanied by increased production of both EGF and TGF-α [Murphy et al., 1988, Lippman et al., 1988] and increases in EGFR levels [Berthois et al., 1989; Bates et al., 1990]. Addition of antibodies to EGFR results in a partial block of E_2 stimulation of growth [Bates et al., 1988]. This finding suggests that although E_2 stimulation of growth is partially due to production of factors interacting with EGFR, other factors are also relevant. A possible role for insulin-like growth factor-1 in this function is presented in the Discussion.

Epidemiological evidence from breast cancer patients also supports a role for EGF, TGF-α, and EGFR in breast cancer. Primary tumor samples have been shown to contain significant levels of TGF-α mRNA and protein [Nickell et al., 1983; Salomon et al., 1984]. Tumors show a large degree of variability in the level of EGFR expressed, with higher EGFR levels in breast cancer biopsies corresponding to significantly poorer prognosis [Spyratos et al., 1990; Nicholson et al., 1991]. This finding is intriguing as studies of normal mammary tissue have shown that the highest levels of EGFR observed in breast cancer biopsies are also observed in normal tissue [Pekonen et al., 1988; Barker et al., 1989]. The best explanation for the correlation between high EGFR levels in breast cancer and poor prognosis is that it is not the absolute level of EGFR that is important, but the interrelationship between EGFR levels and other prognostic factor such as ER status. The highest levels of EGFR are observed in ER^- breast cancers, the hormonally-independent form of the disease, which has the poorest prognosis. This supports the importance of the interrelationship between these two pathways [Toi et al., 1989]. It is possible that the elevation of EGFR levels observed in ER^- mammary tumors represents a key step in their progression to hormone independence. These epidemiological findings are yet another line of evidence supporting the hypothesis that deregulation in growth factor production or responsiveness is likely involved in the progression of breast cancer from a hormonally responsive disease to a hormonally independent disease.

3. Thesis Objectives

Although the literature contains much information on the hormonal and growth factor regulation of mammary gland growth, there is still much that is not understood regarding the effects of E_2, EGF, and TGF-α on the growth of HMEC. The principal objective of this thesis was to investigate the effects of each of these factors, alone and in combination, on HMEC in serum-free primary culture. Although E_2 has been shown to stimulate the growth of MEC growth *in vivo*, it is presently unclear whether E_2 can directly stimulate the proliferation of HMEC in serum-free primary culture. This thesis investigates the growth effects of E_2 at concentrations ranging from 1-1000 nM on the growth of HMEC from reduction mammoplasties, fibroadenomas, and ER^+ carcinomas. The objectives of this portion of the thesis were to determine if E_2 could directly effect the growth of HMEC from either of the three mammary tissue types in serum-free primary culture and to describe any differences in E_2 responses that exist among the different tissue types. Another objective was to determine if factors present in the serum-free medium are involved in modulation of any observed E_2 effects on HMEC growth.

Both EGF and TGF-α have important growth regulatory roles in both normal and malignant mammary tissue. This thesis investigates the effects of both EGF and TGF-α on HMEC from reduction mammoplasties, fibroadenomas, and ER^+ carcinomas. The objective of this portion of the thesis was to determine if these growth factors were able to directly stimulate the growth of HMEC in serum-free primary culture and if there were any significant differences in the responses of HMEC from the three different mammary tissue types. Another objective of this portion of the thesis was to compare the effects of EGF and TGF-α in order to determine if there were any differences in HMEC growth responses to the two factors.

Evidence indicates that E_2 stimulated production of paracrine growth factors and E_2 modulation of HMEC responsiveness to growth factors is likely involved in the E_2 stimulation of

HMEC growth. This thesis investigates the effects of E_2 plus EGF on the growth of HMEC in serum-free primary culture. The objective of this portion of the thesis was to determine if the addition of exogenous EGF would be capable of increasing E_2 stimulation of growth in HMEC in serum-free primary culture.

CHAPTER 2

MATERIALS AND METHODS

1. Sample Procurement and Assessment

Normal tissue was obtained from reduction mammoplasties; fibroadenoma and carcinoma samples were obtained from biopsies and mastectomies. All surgical procedures were performed at local hospitals by collaborating surgeons. All carcinoma samples chosen for this study were ER^+ in situ or infiltrating ductal carcinomas. ER levels and pathology reports were provided by the pathology departments of the hospitals.

2. Tissue Preparation and Freezing

An insulated container equipped with sterile cups each containing transport medium (Appendix 1) on ice was delivered to the operating room on the morning of the surgeries. Samples were aseptically placed in the transport medium by operating room nurses and brought back to the tissue culture room as soon as possible after surgery. Under sterile conditions, excess fat was trimmed from the tissues with scalpels. The remaining tissue was minced into approximately 1 mm^3 pieces using 2 scalpels in a cross-cutting manner. With larger reduction mammoplasty samples it was necessary to change scalpel blades frequently due to dulling of the blades, which lead to difficulty in sufficiently mincing the tissue. Using forceps, the minced tissues from small reduction mammoplasties, biopsies and mastectomies were transferred to 1.7 ml cryotubes until they were 1/2 full. The tube was filled with freezing medium to a volume of 1 ml (Appendix 2). The freezing tubes containing minced tissue and freezing medium were inverted gently to insure mixing of freezing medium and the tissue pieces. The tissue was then slowly frozen and stored in liquid nitrogen until dissociated for cell

culture. Large reduction mammoplasties were dissociated as described below prior to freezing.

3. Dissociation Procedure

Frozen tissue was removed from storage in liquid nitrogen and rapidly thawed by first warming with rotation of the vial between the hands, followed by immersion in a 37^{0}C water bath. The vial was wiped with 70 % ethanol prior to opening. A Kim-wipe wetted with 70 % ethanol was held over the lid to avoid aerosol release upon opening of the tube. The tissue was aseptically transferred from the freezing vial to a 15 ml conical centrifuge tube and 5 ml of DME/F12 (1:1) (Terry Fox Media Preparation Services) + 10 mM Hepes (Sigma) pre-warmed to 37^{0}C was added to the tube. The mixture was spun in a clinical centrifuge for 3 min at 1000 rpm (100 x g). The supernatant was discarded and 5 ml dissociation medium (Appendix 3) was added to the tube. The dissociation procedure has been described previously [Emerman et al., 1990]. The mixture of tissue and dissociation medium was transferred into 250 ml dissociation flasks. The centrifuge tube was rinsed with a further 5 ml of dissociation medium that was also transferred to the dissociation flask. An additional 40 ml of dissociation medium was added to the flask bringing the total volume of dissociation medium to 50 ml. In the smaller samples, such as minced tissue from biopsies, a 125 ml dissociation flask with 25 ml of dissociation medium was used. The dissociation flask was covered in sterile tin foil and parafilm and placed in a gyrating shaker inside a 37^{0}C incubator and shaken for approximately 18 h. Starting at 15 h the dissociation mixture was examined every 1 h and dissociation was considered complete when no large pieces of tissue remained. Typically, the solution was cloudy with stringy appearing aggregates of dissociated cells in suspension. The dissociation solution was divided equally into four 15 ml or 7 ml conical centrifuge tubes, depending on the amount of dissociation medium and dissociated tissue. The cell suspension was centrifuged in a clinical centrifuge for 4 min at 800 rpm (80 x g). This centrifugation speed was chosen to

pellet preferentially the epithelial cells present in the suspension. The supernatant was usually discarded and the cell pellets were combined and resuspended in 10 ml of DME. For experiments utilizing normal fibroblast feeder layers, the supernatant from dissociations of reduction mammoplasty tissues was collected and centrifuged for 4 min at 1000 rpm (100 x g). The supernatant of this centrifugation was discarded and the pellet was resuspended in 10 ml DME, then treated the same as the epithelial cell pellet, described as follows. The cells were resuspended and centrifuged for 4 min at 1000 rpm (100 x g). The supernatant was removed and discarded, and the pellet was again resuspended in 10 ml of DME/F12/Hepes. The solution was again centrifuged for 4 min at 1000 rpm (100 x g) and the pellet resuspended in 5 ml of DME/F12/Hepes. The purpose of the repeated washings was to remove any remaining enzymes from the dissociation medium. To determine cell numbers, 0.1 ml of the cell suspension was removed and placed in a clean 2 ml glass tube. A small drop of trypan blue (pH 7.2) was added to the solution in order to distinguish between viable and dead cells. The plasma membranes of dead cells are not able to prevent trypan blue from entering the cytoplasm, and therefore, the dead cells stain blue. Viable cells were counted on a hemocytometer. For large reduction mammoplasty samples, the total cell yield was calculated and the dissociated cells were mixed with freezing medium at a concentration of 1×10^7 cells / ml , then slowly frozen and stored in liquid nitrogen as described earlier. If the dissociated cells were not to be frozen, they were cultured as described below.

4. Cell Culture

Following the determination of viable cell number, the cells were centrifuged for 3 min at 1000 rpm (100 x g). The supernatant was discarded and the cell pellet resuspended in attachment medium (Appendix 4). The cells were seeded on dehydrated collagen-coated 24 well tissue culture dishes at 3×10^5 cells / cm^2. In the case of fibroblasts grown to be feeder layers, the cells were directly seeded onto plastic. Collagen coated wells were made by

adding of one drop of rat tail collagen (Appendix 5) to each well [Emerman and Wilkinson, 1990]. The dish was swirled gently so that the collagen covered the entire lower surface of the tissue culture well and any excess collagen was removed. The freshly coated dishes were allowed to dry inside the laminar flow hood under UV. light to insure sterility. All cultures were incubated at 37^0C in 95 % air: 5 % CO_2. For the first 24 h of culture, the medium consisted of the attachment medium containing 5 % pooled human serum from normal donors (Appendix 6). This medium allowed the cells to attach to the substrate [Emerman and Wilkinson, 1990]. After 24 h in culture, the medium for cultures of epithelial cells was changed to a phenol-red-free, serum-free medium (Appendix 7) with no extra additives; cultures of fibroblasts were left in the serum-containing medium until they were confluent. It has been observed that fibroblasts in serum-containing media grow rapidly [Emerman and Wilkinson, 1990]. All epithelial cultures remained in the serum-free medium for an additional 24 h to insure maximal removal of serum from the medium. After 24 h, the medium was changed and serum-free medium containing varying amounts of E_2 (1 to 1000 nM) and/or EGF (1 - 100 ng/ml) or TGF-α (1 - 100 ng/ml) were added to the cultures. All growth factors and media supplements (except fetal calf serum, Gibco) were obtained from Sigma and prepared according to manufacturers instructions. It has been observed that the phenol-red used as a pH indicator in tissue culture medium possesses estrogenic activity that might mask the growth effects of exogenously added E_2 [Benthois et al., 1986]. In order to avoid this, all experiments were carried out in phenol-red free medium. The insulin concentration in some experiments was varied from 0.1 to 10.0 μg/ml to determine if high concentrations of insulin were masking an estrogenic effect on growth, as has been reported [van der Burg et al., 1988, Ruedl et al., 1990]. In some studies, a monoclonal antibody to the EGF receptor, MAb 528 (Oncogene Science) , was added to the medium at 1.5 μg/ml to block EGF binding. This MAb binds to the EGF receptor, blocking EGF binding as well as blocking EGF stimulation of the receptor tyrosine-kinase activity [Arteaga et al., 1988]

5. Growth Studies

Media were changed every 3 d and cultures were observed daily using a phase contrast microscope. When the fastest growing cultures were between 70 - 80 % confluent, a final media change was done. The growth assay used has been described previously [Furlanetto and DiCarlo, 1984]. Fourteen h after the last media change, tritiated thymidine (^{3}H-TdR) at 1 μCi/ml was added to each well. After 6 h the media were removed and the cultures were fixed in 10 % trichloroacetic acid (TCA) at 4^{0}C for 15 min. The 10 % TCA was removed and each well was washed 3 x with 5 % TCA at 4^{0}C for 5 min each. The acid-insoluble material was dissolved in 2N NaOH at room temperature for 24 h. Aliquots (250 μl) were removed from each well and transferred into plastic scintillation vials. To each vial 2.75 ml of organic scintillation cocktail was added. Glacial acetic acid (25 μl) was also added to each scintillation vial to neutralize the NaOH solution. The addition of acid is necessary to allow dissolving of the aqueous solvent into the scintillation cocktail. The amount of ^{3}H-TdR incorporation into DNA was measured on a Beckman β-counter. Incorporation was measured in disintegrations per minute (dpm). These values were subsequently converted to percents of controls to allow for comparison of results among experiments. In all cases, the controls were cells grown in the absence of E_2, EGF, and TGF-α. The mean values were compared for significant differences with a two-tailed students T-test for differences between means.

CHAPTER 3

RESULTS

1. The Effects of 17β-estradiol (E_2) on Growth of Human Mammary Epithelial Cells (HMEC) in Primary Culture

Given the evidence for an important role of E_2 in the growth control of MEC *in vivo*, and in and in ER^+ human breast cancer cell lines *in vitro*, the effects of different concentrations of E_2 on the growth of primary cultures of cells from reduction mammoplasties, fibroadenomas, and carcinomas were examined. In all experiments, cells were seeded at 3×10^5 cells/cm^2 in serum containing attachment medium (Appendix 4). After 24 h, the medium was changed to serum-free medium (Appendix 7) plus different concentrations of E_2. When the fastest growing cultures were 70 - 80 % confluent, all cultures were labeled with 1 μCi/ml ^{3}H-TdR for 4 h prior to termination. In all experiments, growth was assessed by the level of incorporation of ^{3}H-TdR relative to the control condition which was the serum-free medium with no added E_2.

Two separate experiments utilizing cultures of HMEC from fibroadenomas were carried out to determine the optimal time after the last medium change to assay growth using the ^{3}H-TdR incorporation assay. The results of these experiments are shown in Figures 1a and 1b. In both experiments, it was observed that reasonable incorporation of ^{3}H-TdR occurred between 12 and 16 hours after a medium change. For this reason, cell growth assays were done 14 h after the last medium change. There was no E_2 stimulation of growth in cells from either sample at any of the time points examined. The growth of cells from FA 49 was not significantly ($p < 0.05$) effected by either concentration of E_2 at any of the timepoints examined. Growth of cells from FA 50 was not significantly effected at the 8 h timepoint; however, the inhibition of growth in FA 50 by 10 nM E_2 at 12 and 16 h, and by 1000 nM E_2 at 16 h were all statistically significant ($p < 0.05$).

Figure 1 : The effects of time after the last medium change on the incorporation of ^{3}H-TdR into primary cultures of HMEC from 2 fibroadenomas (Fig. 1a. FA 49. Fig. 1b. FA 50).

Cells were cultured in serum-free medium containing different concentrations of E_2. The values shown for ^{3}H-TdR incorporation are the means ± SEM of triplicate wells in the same condition. The level of ^{3}H-TdR incorporation is dependent on the time at which the ^{3}H-TdR is added after the last medium change. High level of incorporation were observed at 12 and 16 h. At none of the concentrations examined was there any effect of E_2 on the incorporation of ^{3}H-TdR into FA 49 (Fig. 1a.). E_2 at both 10 and 1000 nM dramatically reduced incorporation of ^{3}H-TdR into the cells at all three timepoints, however, the inhibition of incorporation was only statistically significant ($p < 0.05$) at the 12 and 16 h timepoint in FA 50 (Fig. 1b.).

Figure 1a.

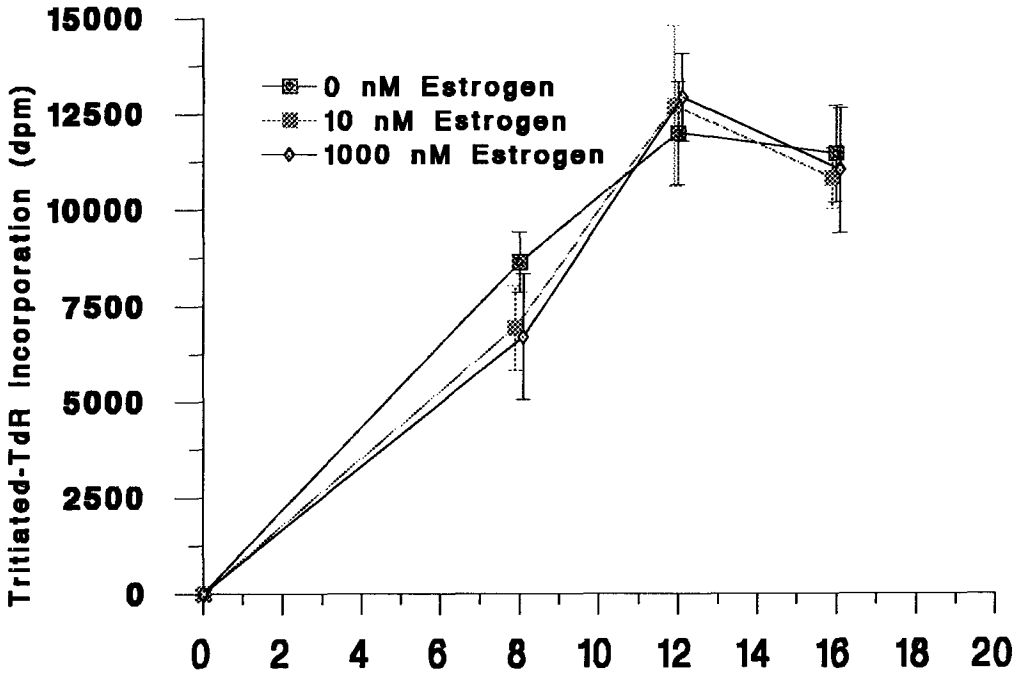
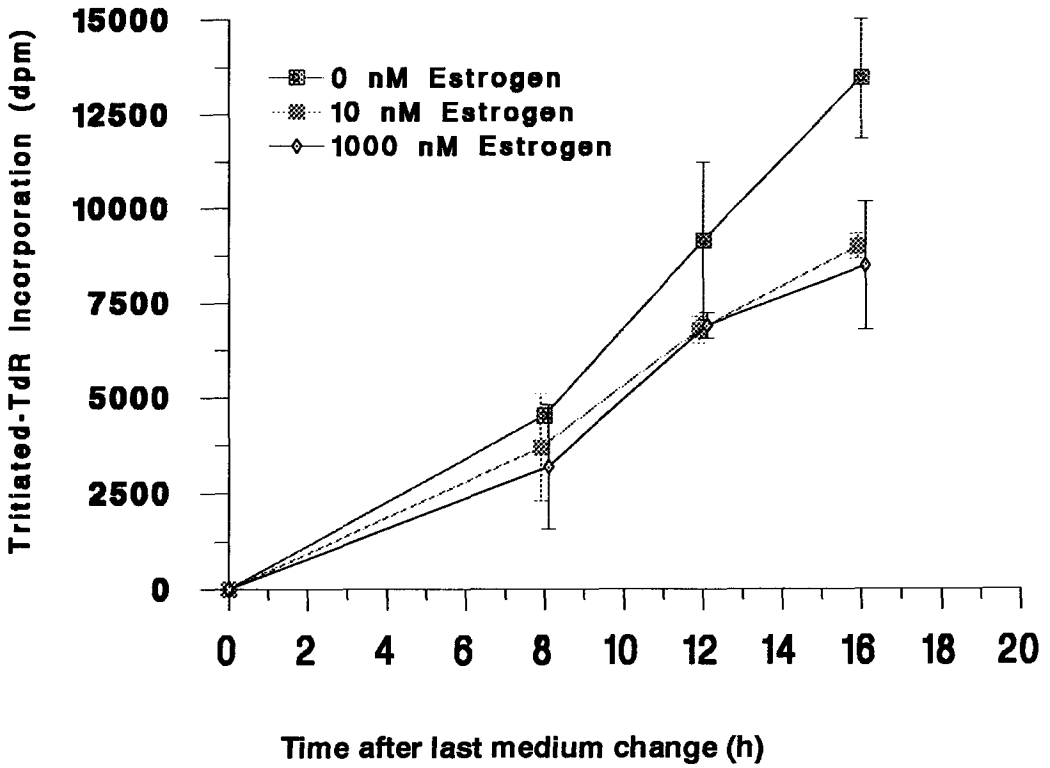


Figure 1b.



1a. The Effects of E_2 on the Growth of Primary Cultures of Normal HMEC Obtained From Reduction Mammoplasties

The effects of different concentrations of E_2 on the growth of cultured cells from reduction mammoplasties are summarized in Table I. Also included in Table I are the results of experiments examining the effects of E_2 on the growth of MCF-7 breast cancer cells. These experiments served as a positive control for estrogen activity as MCF-7 are well characterized as being growth stimulated by E_2 at the concentrations examined [Lippman and Dickson, 1989]. In none of the 8 experiments examining the effect of E_2 at concentrations ranging from 1 to 1000 nM was any significant stimulation of growth of the primary cultures of HMEC observed. However, in 3 of 8 experiments there was a significant inhibition ($p < 0.05$) of growth. Inhibition of growth was generally observed at the high (pharmacological) concentrations of E_2, but was also seen at the lower (physiological) concentrations of E_2 in a few individual cases. Examples of the inhibitory effects of E_2 on the growth of HMEC from reduction mammoplasties are presented in Figure 2.

Two experiments examined the effects of E_2 on the growth of normal HMEC at different times *in vitro* and the results are presented in Figures 3a and 3b. In both experiments, the number of days *in vitro* had no significant effect on E_2 regulation of cell growth. However, the SEM increased with time in culture. Cell growth was therefore ideally measured as early as possible in an attempt to minimize the variation within individual conditions.

Table I : The effects of 17β-estradiol (E_2) on the growth of primary cultures of HMEC from reduction mammoplasties.

	Concentration of E_2 (nM)				
Specimen no. (div)[a]	**0**	**1**	**10**	**100**	**1000**
Redn 7 (5 div)	100 ± 5[b]	95 ± 3	100 ± 5	109 ± 15	115 ± 24
Redn 8 (5 div)	100 ± 3	90 ± 11	68 ± 13*	75 ± 8*	60 ± 10*
Redn 9 (6 div)	100 ± 7	98 ± 12	84 ± 17	91 ± 8	82 ± 8
Redn 10 (6 div)	100 ± 20	112 ± 33	97 ± 11	101 ± 11	92 ± 7
Redn 11 (7 div)	100 ± 9	90 ± 11	92 ± 12	63 ± 19*	69 ± 18
Redn 12 (8 div)	100 ± 22	N.D.[c]	80 ± 26	N.D.	80 ± 11
Redn 13 (8 div)	100 ± 7	72 ± 9*	60 ± 5*	N.D.	62 ± 6*
Redn 14 (9 div)	100 ± 27	98 ± 6	77 ± 4	N.D.	83 ± 3
MCF-7	100 ± 9		174 ± 16		126 ± 6
Summary	**100 ± 12** **n=8[d]**	**91 ± 12** **n=7**	**85 ± 12** **n=8**	**85 ± 12** **n=5**	**80 ± 11** **n=8**

a : div = days *in vitro*
b : Values shown represent a percentage of growth relative to the control (control = 0 nM estrogen). Values are expressed as the mean ± SEM of each condition done in triplicate.
c : N.D. = no data for these conditions due to limitations in cell numbers from various samples
d : n = number of individual samples examined in each condition
* significant inhibition of growth ($P < 0.05$)

Figure 2 : The effects of 17β-estradiol (E_2) on the growth of primary cultures of human mammary epithelial cells (HMEC) from 2 reduction mammoplasties (Redn 10 & Redn 11).

Growth was assessed by the incorporation of ^{3}H-TdR (1 μCi/ml) into the cells over a 4 h period. The absolute levels of incorporation have been converted to % of control values, with the level of incorporation into the control condition (0 nM E_2 in the serum-free medium, Appendix 7) being assigned a value of 100 %. Values shown represent the mean ± SEM of triplicate wells of the same condition. E_2 caused no significant effects on the growth of Redn 10, however a significant ($p < 0.05$) inhibition of growth of Redn 11 is shown at 100 nM E_2. The inhibition at 1000 nM E_2 in Redn 11 is not statistically significant.

Figure 2.

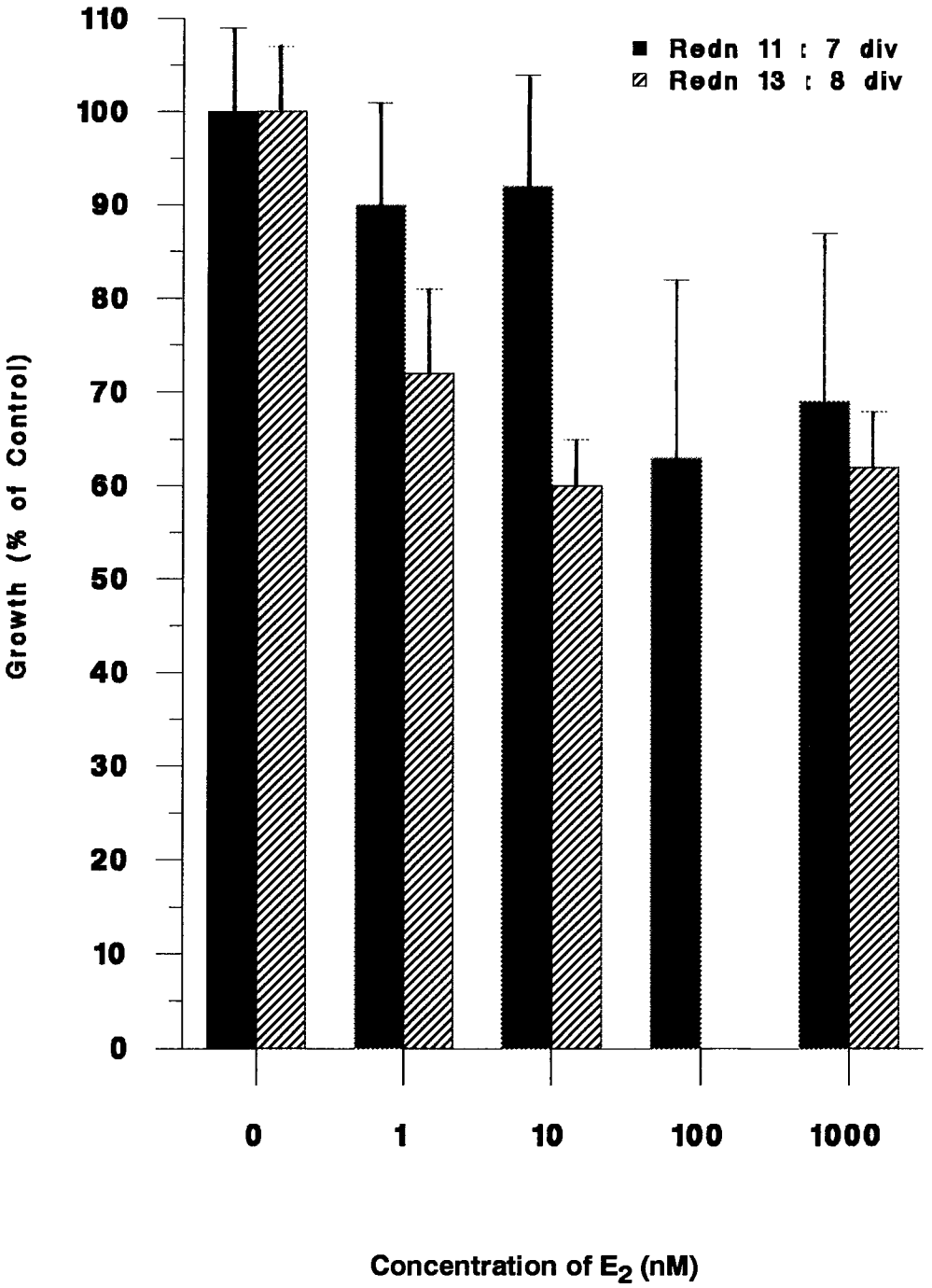

Redn 11 : 7 div
Redn 13 : 8 div
Growth (% of Control)
110
100
90
80
70
60
50
40
30
20
10
0
0
1
10
100
1000
Concentration of E_2 (nM)

Figure 3 : The effect of time in culture on E_2 regulation of growth in primary cultures of HMEC from 2 reduction mammoplasties (3a. Redn 8., 3b. Redn 9).

Growth was assessed by the incorporation of ^{3}H-TdR (1 μCi/ml) into the cells over a 4 h period. The absolute levels of incorporation have been converted to % of control values, with the level of incorporation into the control condition (0 nM E_2 in the serum-free medium, Appendix 7) being assigned a value of 100 %. Values shown represent the mean ± SEM of triplicate wells of the same condition. Growth of Redn 8 was significantly ($p < 0.05$) inhibited by 10, 100 and 1000 nM E_2 at 5 div (Fig. 3a.). Due to the considerably higher SEM values at 14 div, the inhibitory effects of E_2 are not significant at any of the concentrations examined. Growth of Redn 9 was significantly ($p < 0.05$) inhibited by 1000 nM E_2 at 6 div, however, there were no significant effects of E_2 at any concentration on the growth of Redn 9 on either 8 or 11 div (Fig. 3b.).

Figure 3a.

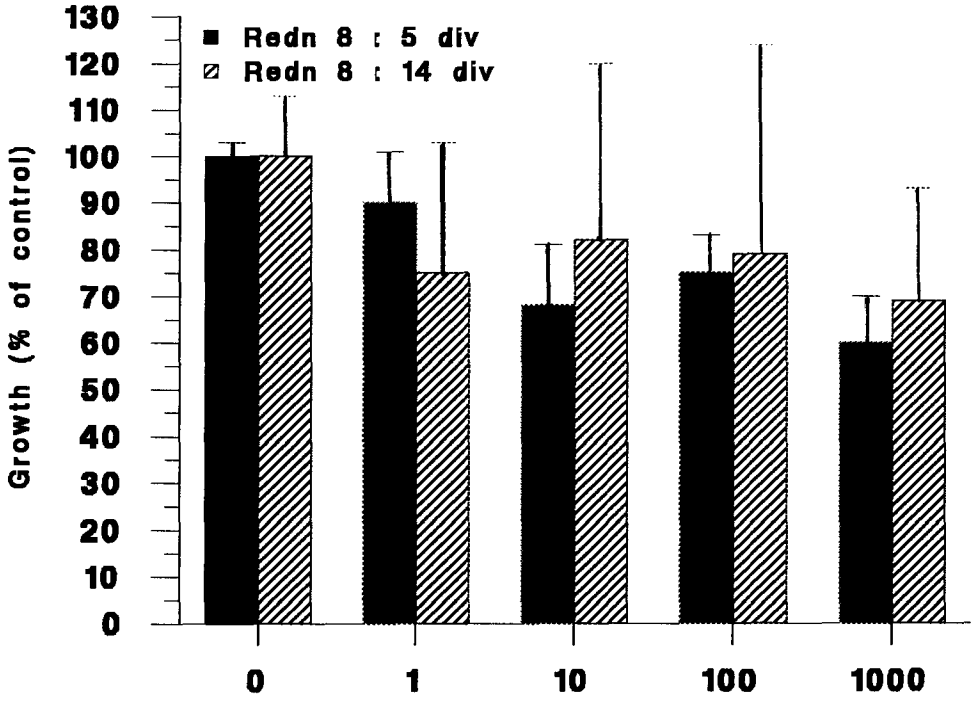
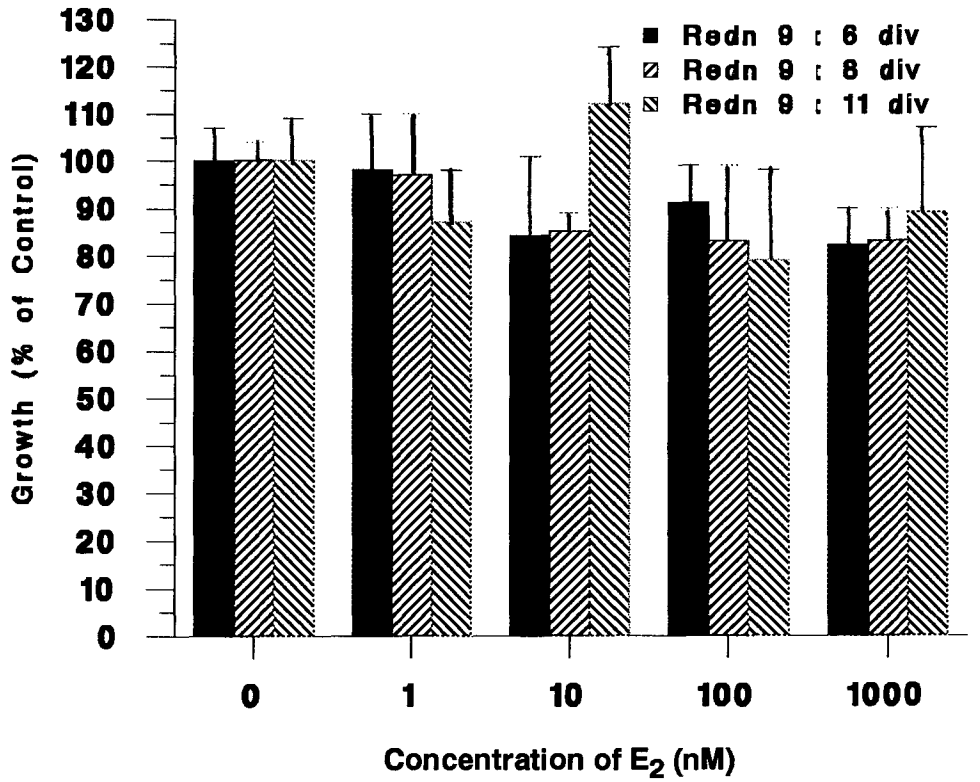


Figure 3b.



1b. The Effects of E_2 on the Growth of Primary Cultures of HMEC Obtained from Fibroadenomas

The effects of different concentrations of E_2 on the growth of HMEC from fibroadenomas, a type of benign breast disease, are shown in Table II. In none of 19 samples was a significant stimulation of growth by E_2 observed at any of the concentrations used. In a total of 5 of 10 samples used to examine the effects of E_2 at 1000 nM, a significant ($p < 0.05$) inhibition of growth was observed. The results of two representative experiments demonstrating these effects are presented in Figure 4.

1c. The Effects of E_2 on the Growth of Primary Cultures of HMEC from ER^+ Mammary Carcinomas

The effects of E_2 on the growth of HMEC from ER^+ mammary carcinomas were examined in 12 samples and the results are shown in Table III. In none of the 12 carcinoma samples examined was a significant stimulation of growth by E_2 observed at any of the concentrations examined. Significant inhibition of growth by E_2 at 1000 nM was observed in 4 of 8 samples. The results of two individual experiments demonstrating these effects are presented in Figure 5.

1d. Summary of the Effects of E_2 on the Growth of HMEC in Serum-Free Primary Culture

A summary of all the experiments examining the effects of E_2 on the growth of HMEC from the 3 types of tissue is presented in Table IV. E_2 failed to stimulate the growth of any of the cultures at all concentrations examined. There is no significant differences between the different mammary tissue types. However, there is a trend towards inhibition of growth at the higher concentrations of E_2.

Table II : The effects of 17β-estradiol (E_2) on the growth of primary cultures of HMEC from fibroadenomas

	Concentration of E_2 (nM)				
Specimen no. (div)	**0**	**1**	**10**	**100**	**1000**
FA 43 (8 div)[a]	100 ± 17[b]	N.D.[c]	146 ± 26	N.D.	N.D.
FA 45 (8 div)	100 ± 8	N.D.	104 ± 15	N.D.	N.D.
FA 47 (8 div)	100 ± 21	92 ± 24	81 ± 23	39 ± 15*	31 ± 6*
FA 48 (9 div)	100 ± 15	93 ± 31	156 ± 44	130 17	113 ± 46
FA 49 (7 div)	100 ± 10	N.D.	94 ± 7	N.D.	96 ± 14
FA 50 (10 div)	100 ± 12	N.D.	66 ± 2*	N.D.	63 ± 12*
FA 51 (11 div)	100 ± 9	N.D.	129 ± 17	N.D.	N.D.
FA 52 (8 div)	100 ± 37	101 ± 22	102 ± 20	92 ± 7	76 ± 30
FA 54 (9 div)	100 ± 11	N.D.	81 ± 30	N.D.	80 ± 25
FA 58 (9 div)	100 ± 22	N.D.	136 ± 27	N.D.	N.D.
FA 59 (9 div)	100 ± 16	N.D.	125 ± 16	N.D.	N.D.
FA 60 (8 div)	100 ± 14	N.D.	101 ± 10	N.D.	100 ± 4
FA 62 (11 div)	100 ± 40	N.D.	160 ± 35	N.D.	44 ± 29
FA 63 (11 div)	100 ± 8	N.D.	82 ± 6*	N.D.	N.D.
FA 65 (11 div)	100 ± 4	N.D.	89 ± 2*	N.D.	N.D.
FA 67 (11 div)	100 ± 15	N.D.	60 ± 4*	N.D.	61 ± 5*
FA 81 (7 div)	100 ± 28	N.D.	110 ± 25	N.D.	N.D.
FA 82 (7 div)	100 ± 17	N.D.	74 ± 6	N.D.	N.D.
FA 86 (9 div)	100 ± 20	N.D.	111 ± 18	N.D.	92 ± 22
Summary	**100 ± 17 n = 18[d]**	**95 ± 25 n = 3**	**105 ± 18 n = 18**	**90 ± 13 n = 3**	**74 ± 21 n = 9**

a : div = days *in vitro*
b : Values shown represent a percentage of growth relative to the control (control = 0 nM estrogen). Values are expressed as the mean ± SEM of each condition done in triplicate.
c : N.D. = no data for these conditions due to limitations in cell numbers from various samples
d : n = number of individual samples examined in each condition
* significant inhibition of growth ($P < 0.05$)

Table III : The effect of 17β-estradiol (E_2) on the growth of primary cultures of HMEC from carcinomas.

	Concentration of E_2 (nM)			
Specimen no. (div)[a]	**0**	**10**	**100**	**1000**
HMC 96 (13 div)	100 ± 16[b]	93 ± 17	N.D.	82 ± 13
HMC 97 (13 div)	100 ± 5	N.D.[c]	92 ± 10	N.D.
HMC 101 (11 div)	100 ± 15	114 ± 23	74 ± 35	48 ± 7*
HMC 102 (11 div)	100 ± 39	92 ± 1	59 ± 19	53 ± 2*
HMC 107 (11 div)	100 ± 58	37 ± 8	N.D.	33 ± 4*
HMC 108 (13 div)	100 ± 23	57 ± 21	N.D.	84 ± 38
HMC 109 (12 div)	100 ± 40	130 ± 29	N.D.	N.D.
HMC 110 (12 div)	100 ± 10	139 ± 21	N.D.	N.D.
HMC 111 (13 div)	100 ± 38	112 ± 19	N.D.	105 ± 17
HMC 127 (15 div)	100 ± 15	120 ± 24	N.D.	N.D.
HMC 129 (13 div)	100 ± 26	89 ± 24	N.D.	81 ± 16
HMC 130 (13 div)	100 ± 7	56 ± 10*	N.D.	27 ± 4*
Summary	**100 ± 24 n=12[d]**	**94 ± 18 n=11**	**73 ± 21 n=3**	**64 ± 14 n=8**

a : div = days *in vitro*
b : Values shown represent a percentage of growth relative to the control (control = 0 nM estrogen). Values are expressed as the mean ± SEM of each condition done in triplicate
c : N.D. = no data for these conditions due to limitations in cell numbers from various samples
d : n = number of individual samples examined in each condition
* significant inhibition of growth ($P < 0.05$)

Figure 4 : The effects of E_2 on the growth of primary cultures of HMEC from 2 fibroadenomas (FA 47 & 67).

Growth was assessed by the incorporation of ^{3}H-TdR (1 μCi/ml) into the cells over a 4 h period. The absolute levels of incorporation have been converted to % of control values, with the level of incorporation into the control condition (0 nM E_2 in the serum-free medium, Appendix 7) being assigned a value of 100 %. Values shown represent the mean ± SEM of triplicate wells of the same condition. E_2 at 100 and 1000 nM concentrations significantly ($p < 0.05$) inhibited the growth of FA 47, whereas E_2 at 10 and 1000 nM significantly ($p < 0.05$) inhibited the growth of FA 67.

Figure 4.

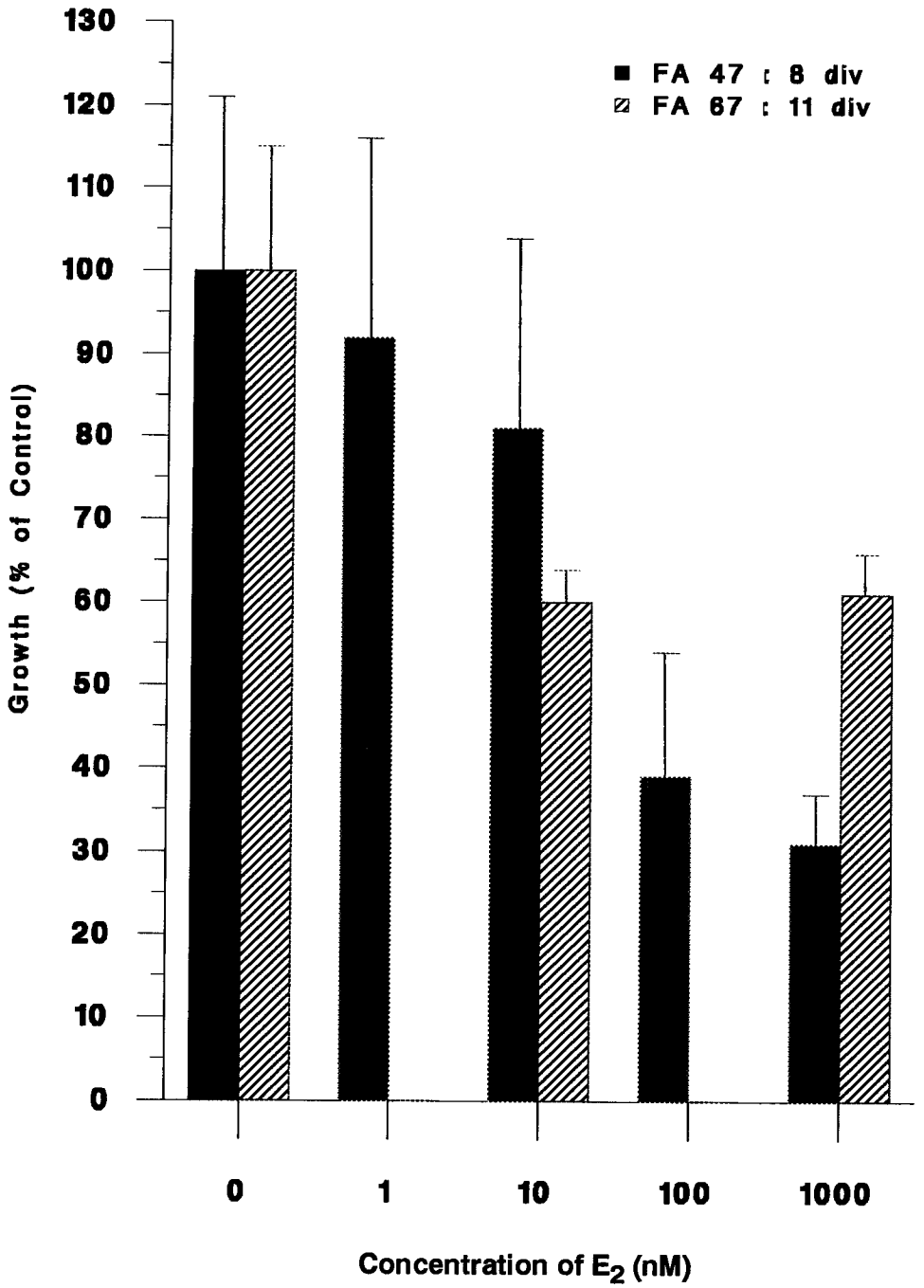

130
120
110
100
90
80
70
60
50
40
30
20
10
0
Growth (% of Control)
FA 47 : 8 div
FA 67 : 11 div
0
1
10
100
1000
Concentration of E_2 (nM)

Figure 5 : The effect of E_2 on the growth of primary cultures of HMEC from 2 ER+ mammary carcinomas (HMC 101 and 102).

Growth was assessed by the incorporation of ^{3}H-TdR (1 μCi/ml) into the cells over a 4 h period. The absolute levels of incorporation have been converted to % of control values, with the level of incorporation into the control condition (0 nM E_2 in the serum-free medium, Appendix 7) being assigned a value of 100 %. Values shown represent the mean ± SEM of triplicate wells of the same condition. E_2 at 1000 nM caused a significant ($p < 0.05$) inhibition of the growth of both HMC 101 and HMC 102.

Figure 5.

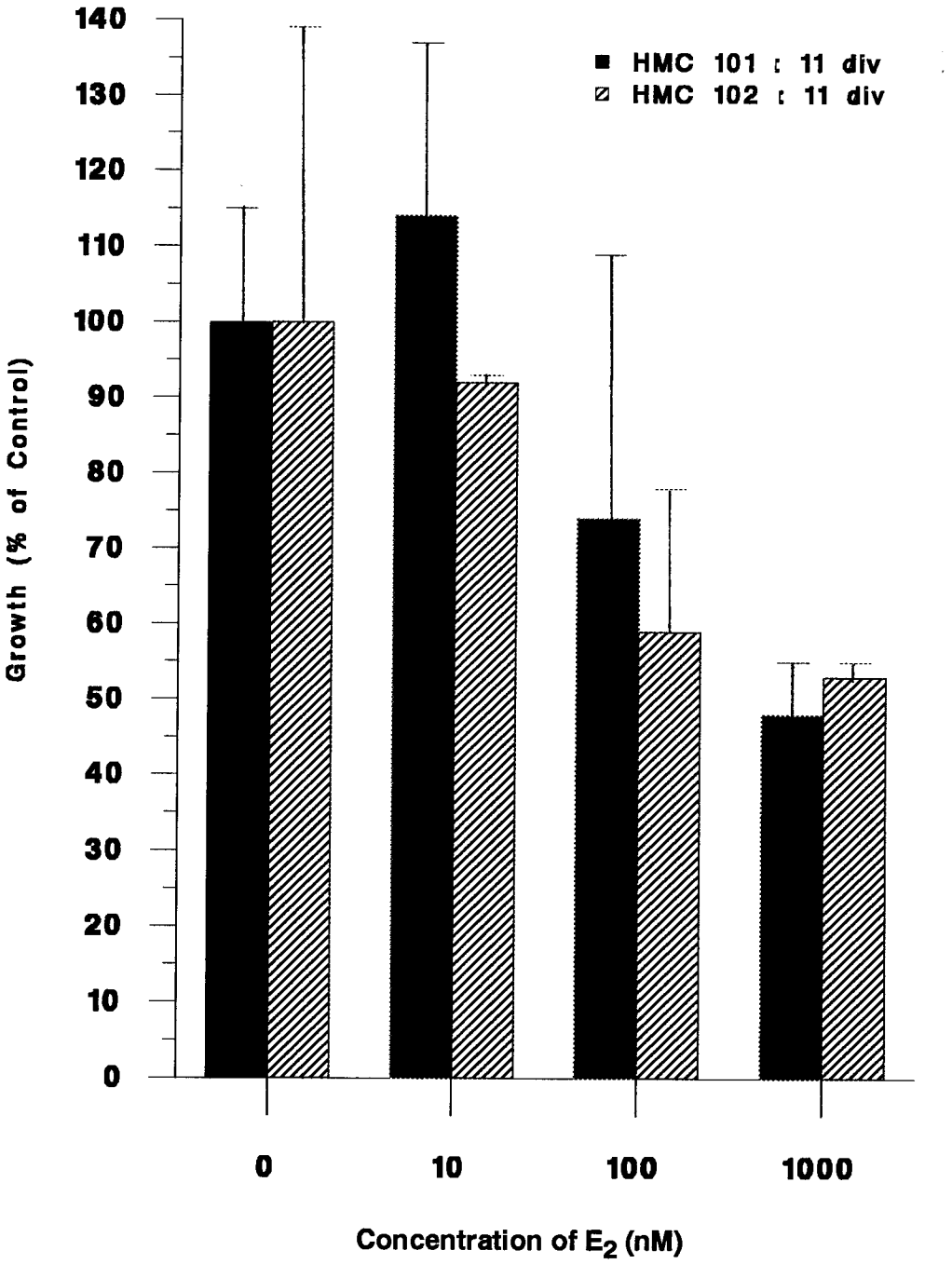

140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
Growth (% of Control)
HMC 101 : 11 div
HMC 102 : 11 div
0
10
100
1000
Concentration of E_2 (nM)

Table IV : Summary of the effects of 17β-estradiol (E_2) on the growth of primary cultures of HMEC from the 3 mammary tissue types

	Concentration of E_2 (nM)				
Tissue Type	**0**	**1**	**10**	**100**	**1000**
Reduction Mammoplasties	100 ± 12[a] n = 11[b]	91 ± 12 n = 10	85 ± 12 n = 11	85 ± 12 n = 8	80 ± 11 n = 11
Fibroadenomas	100 ± 17 n = 19	95 ± 25 n = 3	105 ± 18 n = 19	90 ± 13 n = 3	74 ± 21 n = 10
Carcinomas	100 ± 24 n = 12	N.D.[c]	94 ± 18 n = 11	73 ± 21 n = 3	64 ± 14 n = 8
Summary	**100 ± 18** **n = 42**	**92 ± 15** **n = 13**	**97 ± 16** **n = 41**	**84 ± 14** **n = 14**	**74 ± 15** **n = 29**

a : Values shown represent a percentage of growth relative to the control (control = 0 nM estrogen). Values are the average of individual experiments, and are expressed as the mean ± SEM of each tissue type

b : n = number of individual samples examined in each condition

c : N.D. = no data for these conditions due to limitations in cell numbers from various samples

1e. The Effects of Insulin on E_2 Modulation of HMEC Growth in Primary Cultures

To determine if the high concentration (10 μg/ml) of insulin in our serum-free medium was masking a growth stimulation by E_2, 8 experiments comparing the growth effects of E_2 in serum-free medium with 10 μg/ml of insulin and medium with 0.1 μg/ml of insulin were conducted using HMEC from 7 fibroadenomas and a carcinoma. Two additional experiments investigated the effects of insulin on the growth of HMEC from carcinoma samples, however, insufficient cell numbers did not allow examination of the effects of E_2 in these cultures. The results of these experiments are presented in Table V. In all of the cases examined, the reduction in the concentration of insulin significantly ($p < 0.05$) reduced the growth of cultures of HMEC from both fibroadenomas and carcinomas. Reduction in growth ranged from 33% to 93%, the average reduction being 59%. The effects of E_2 in low concentrations of insulin were generally the same as the effects in high concentrations of insulin. There was no significant stimulation of growth in 7 of the 8 cases examined. Cells from 3 fibroadenomas, FA 63, FA 65, and FA 67, were growth inhibited by E_2 in medium containing 10 μg/ml insulin. However, in cells from FA 67 a stimulation of growth was observed in response to the same concentration of E_2 in 0.1 μg/ml insulin (Figure 6), that inhibited growth in 10 μg/ml insulin, as already discussed.

Table V : The effects of insulin and 17β-estradiol (E_2) on the growth of primary cultures of HMEC from fibroadenomas and carcinomas.

Specimen no. (div)	Concentration of insulin (μg/ml)	Concentration of E_2 (nM) 0	10	1000
FA 58 (9 div)[a]	10	100 ± 22[b]	136 ± 27	N.D.[c]
	0.1	67 ± 8	44 ± 10	N.D.
FA 59 (9 div)	10	100 ± 16	125 ± 16	91 ± 21
	0.1	51 ± 6	63 ± 2	N.D.
FA 60 (8 div)	10	100 ± 14	101 ± 10	100 ± 4
	0.1	56 ± 14	46 ± 8	52 ± 13
FA 62 (11 div)	10	100 ± 40	160 ± 35	44 ± 29
	0.1	17 ± 1	18 ± 4	19 ± 4
FA 63 (11 div)	10	100 ± 8	82 ± 6*	N.D.
	0.1	7 ± 2	4 ± 3	N.D.
FA 65 (11 div)	10	100 ± 4	89 ± 3*	N.D.
	0.1	68 ± 15	50 ± 3	N.D.
FA 67 (11 div)	10	100 ± 13	60 ± 4*	61 ± 5*
	0.1	35 ± 1	48 ± 5**	54 ± 4**
HMC 127 (15 div)	10	100 ± 15	120 ± 24	N.D.
	0.1	41 ± 8	30 ± 11	N.D.
HMC 140 (11 div)	10	100 ± 13	N.D.	N.D.
	0.1	38 ± 17	N.D.	N.D.
HMC 141 (13 div)	10	100 ± 14	N.D.	N.D.
	0.1	31 ± 2*	N.D.	N.D.
Summary	**10 μg/ml**	**100 ± 16**	**107 ± 16**	**54 ± 15 (n=4)**
	0.1μg/ml	**41± 7 (n=10)[d]**	**39 ± 6 (n=8)**	**42 ± 7 (n=3)**

a : div = days *in vitro*

b : Values shown represent a percentage of growth relative to the control (control = 0 nM estrogen). Values are expressed as the mean ± SEM of each condition done in triplicate

c : N.D. = no data for these conditions due to limitations in cell numbers from various samples

d : n = number of individual samples examined in each condition

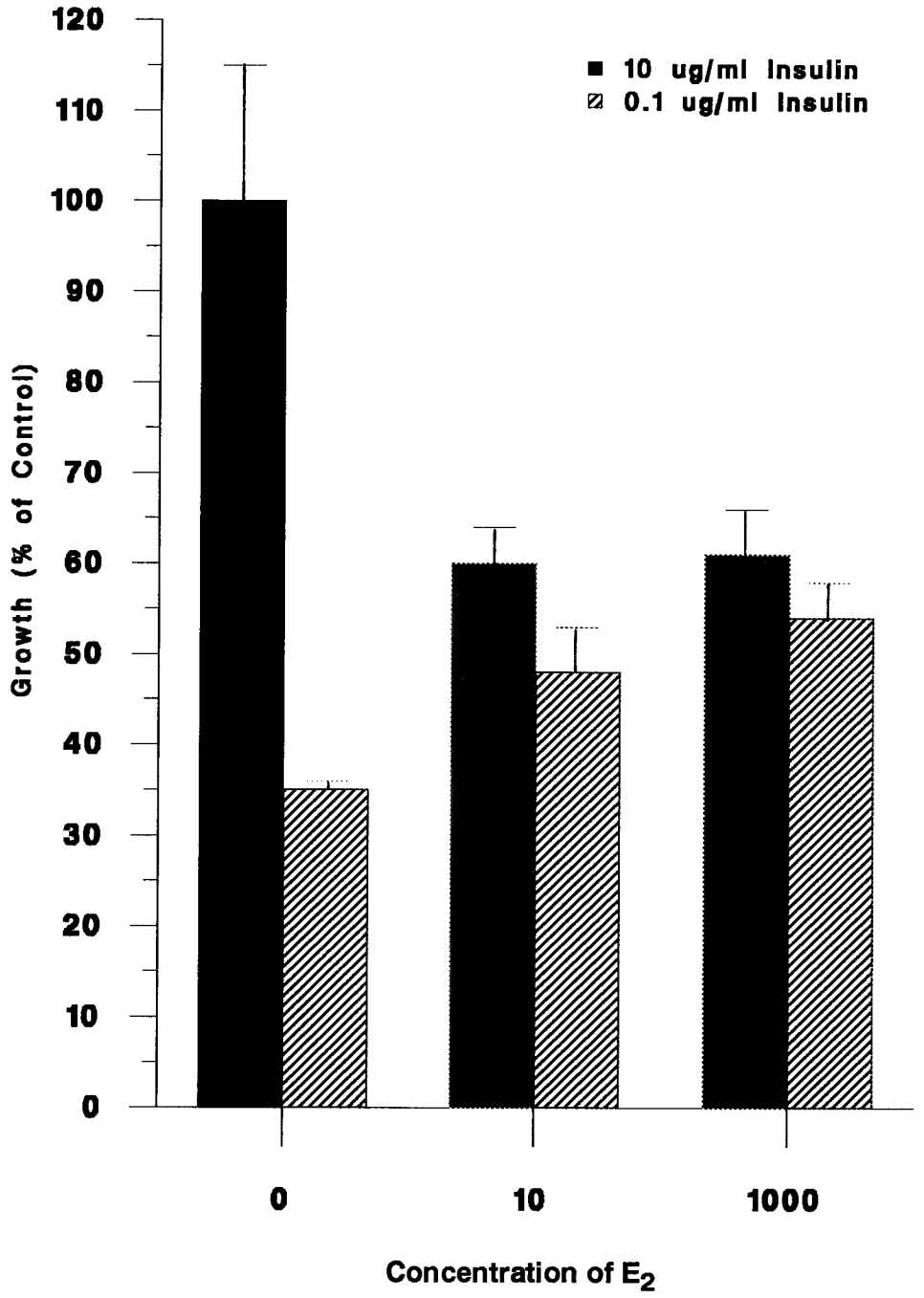
* significant inhibition of growth ($P < 0.05$)

** significant stimulation of growth compared to 0.1 ng/ml insulin with 0 nM estrogen ($p < 0.05$)

Figure 6 : The effects of E_2 and insulin on the growth of primary cultures of HMEC from a fibroadenoma (FA 67).

Growth was assessed by the incorporation of ^{3}H-TdR (1 μCi/ml) into the cells over a 4 h period. The absolute levels of incorporation have been converted to % of control values, with the level of incorporation into the control condition (0 nM E_2 in the serum-free medium, Appendix 7) being assigned a value of 100 %. Values shown represent the mean ± SEM of triplicate wells of the same condition. In medium containing 10 μg/ml insulin, E_2 at 10 and 1000 nM caused a significant inhibition ($p < 0.05$) of growth in FA 67. In medium containing 0.1 μg/ml insulin, E_2 at 10 and 1000 nM caused a significant stimulation of growth compared to 0 nM E_2 in 0.1 μg/ml medium.

Figure 6.


120
110
100
90
80
70
60
50
40
30
20
10
0
Growth (% of Control)
10 ug/ml Insulin
0.1 ug/ml Insulin
0
10
1000
Concentration of E_2

2. The Effects of Epidermal Growth Factor (EGF) and Transforming Growth Factor-α (TGF-α) on the Growth of HMEC in Primary Culture

As discussed in the introduction, EGF and TGF-α have been shown to be involved in the direct control of both normal rodent MEC growth *in vivo* (EGF) and human breast cancer cell line growth *in vitro* (EGF and TGF-α). For this reason the effects of these two growth factors on the growth of HMEC in serum-free primary culture were examined.

2a. The Effects of EGF on the Growth of Primary Cultures of HMEC from Reduction Mammoplasties

The effects of different concentrations of EGF on the growth of HMEC from 3 reduction mammoplasties were examined and the results are shown in Figure 7. In all cases, EGF significantly ($p < 0.05$) stimulated growth in a dose-dependent manner. Stimulation ranged from 188% to 1698% of the growth in control cultures without EGF (average stimulation of growth by EGF was 894 % of controls). Although stimulation of growth was seen at all concentrations examined, ranging from 1 to 100 ng/ml of EGF, peak stimulation of growth was observed between 5 - 10 ng/ml. Based on these findings, a value of 10 ng/ml was chosen to study the effects of EGF on HMEC in subsequent experiments.

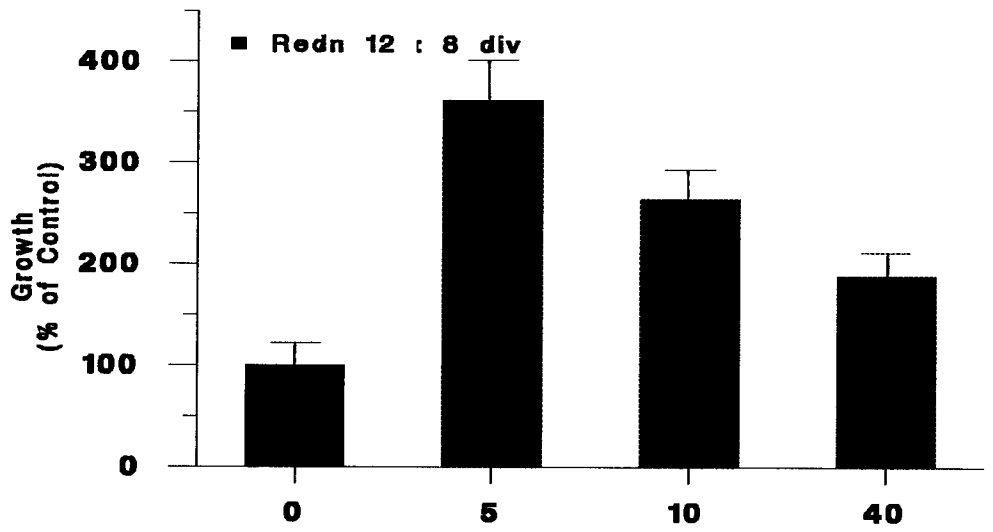
2b. The Effects of EGF and TGF-α on the Growth of Primary Cultures of HMEC From Fibroadenomas

HMEC from 12 fibroadenomas were examined for the effects of EGF on growth. In all cases, EGF was found to stimulate growth significantly ($p < 0.05$). The results of these experiments are shown in Table VI. The degree of growth stimulation ranged from 133% to 3455% (average stimulation of growth was 923%) of the levels observed in control cultures grown without EGF. The growth effects of TGF-α were compared to those of EGF in cultures of HMEC from 6 fibroadenomas (Table VI).

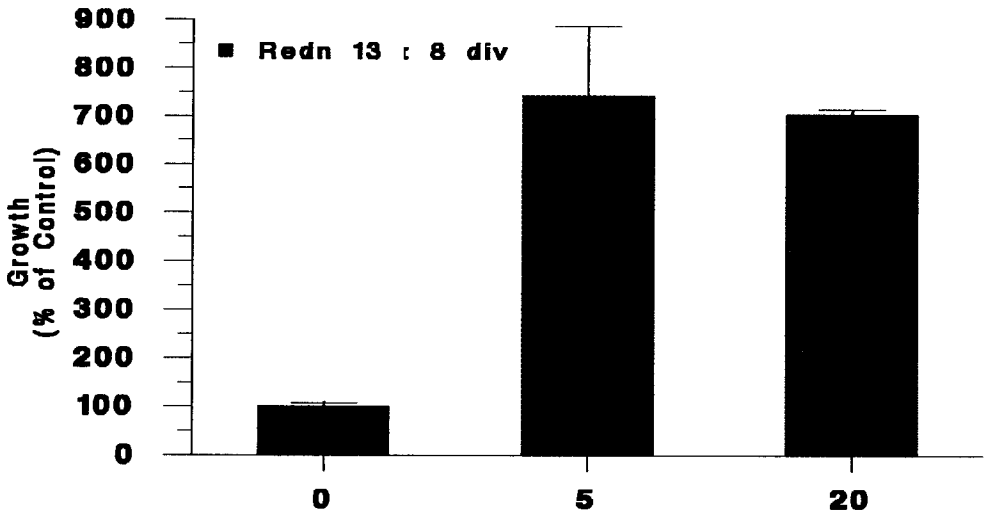
Figure 7 : The effects of EGF on the growth of primary cultures of HMEC from 3 reduction mammoplasties (Redn 12, 13 and 14).

Growth was assessed by the incorporation of ^{3}H-TdR (1 μCi/ml) into the cells over a 4 h period. The absolute levels of incorporation have been converted to % of control values, with the level of incorporation into the control condition (0 ng/ml EGF in the serum-free medium, Appendix 7) being assigned a value of 100 %. Values shown represent the mean ± SEM of triplicate wells of the same condition. EGF significantly ($p < 0.05$) stimulated growth in a dose-dependent manner (1-100 ng/ml). Peak stimulation of growth was observed between 5 - 10 ng/ml.

7a.


Redn 12 : 8 div
Growth (% of Control)
400
300
200
100
0
0
5
10
40

7b.


Redn 13 : 8 div
Growth (% of Control)
900
800
700
600
500
400
300
200
100
0
0
5
20

7c.

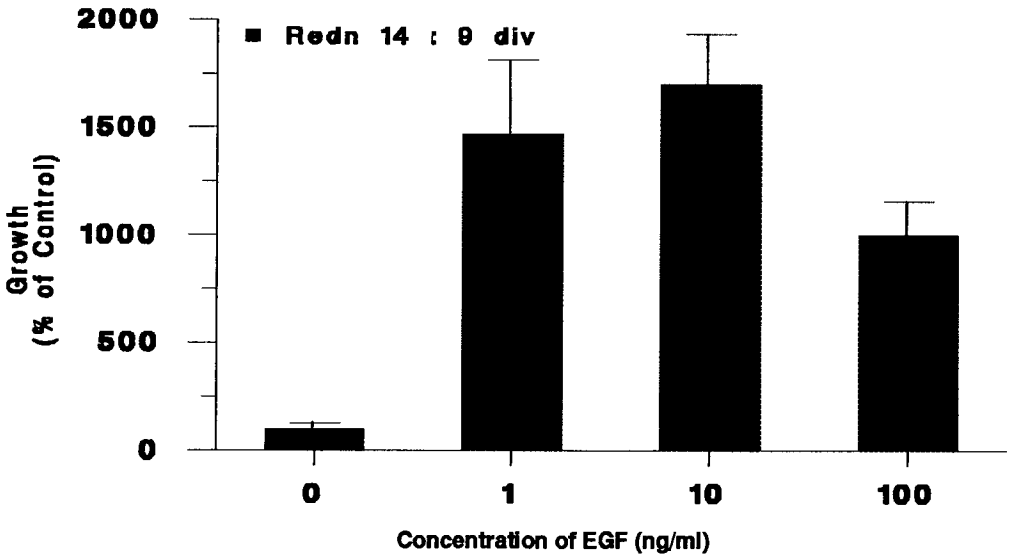

Redn 14 : 9 div
Growth (% of Control)
2000
1500
1000
500
0
0
1
10
100
Concentration of EGF (ng/ml)

Table VI : The effects of EGF and TGF-α on the growth of primary cultures of HMEC from fibroadenomas

Specimen no. (div)	Culture conditions		
	Control	+ EGF (10 ng/ml)	+ TGF-α (10 ng/ml)
FA 43 (8 div)[a]	100 ± 17[b]	167 ± 43	N.D.[c]
FA 45 (8 div)	100 ± 8	197 ± 15*	N.D.
FA 51 (11 div)	100 ± 9	1047 ± 283*	N.D.
FA 52 (8 div)	100 ± 37	188 ± 22*	N.D.
FA 54 (9 div)	100 ± 11	753 ± 161*	2006± 57**
FA 61 (8 div)	100 ± 17	284 ± 67*	394 ± 12*
FA 74 (11 div)	100 ± 6	1616 ± 182*	3984 ± 181**
FA 80 (11 div)	100 ± 23	589 ± 99*	1113 ± 119**
FA 81 (7 div)	100 ± 28	3455 ± 583*	N.D.
FA 82 (7 div)	100 ± 17	2203± 284*	N.D.
FA 84 (13 div)	100 ± 19	583 ± 58*	392 ± 32*
FA 85 (13 div)	100 ± 16	615 ± 109*	543* ± 4
SUMMARY	**100 ± 17 (n=12)[d]**	**923 ± 159 (n=12)**	**1088 ± 63(n=6)**

a : div = days *in vitro*
b : Values shown represent a percentage of growth relative to the control (control = 0 added growth factor). Values are expressed as the mean ± SEM of each condition done in triplicate
c : N.D. = no data for these conditions due to limitations in cell numbers from various samples
d : n = number of individual samples examined in each condition
* significant stimulation of growth compared to control with 0 ng/ml growth factor ($p < 0.05$)
** growth significantly greater than corresponding + EGF condition ($p < 0.05$)

TGF-α was found to be significantly ($p < 0.05$) growth stimulating in all cultures examined. Although individual samples varied greatly in response to TGF-α , TGF-α was equal to, or greater than EGF in stimulating growth of HMEC from fibroadenomas. Stimulation of growth ranged from 392% to 3984% (average was 1088%) of control cultures, which contained no added growth factor. A difference in the dose responses between EGF and TGF-α was also observed in 2 experiments comparing their effects on HMEC growth (Figure 8a and 8b). TGF-α appeared to be active over a lower concentration range, as indicated by the drop in stimulation of HMEC growth at 100 ng/ml compared to 10 ng/ml. In contrast to TGF-α, EGF effects were similar or greater at 100 ng/ml than at 10 ng/ml of EGF.

2c. The Effects of EGF and TGF-α on the Growth of Primary Cultures of HMEC From Carcinomas

Primary cultures of HMEC from 9 carcinomas were examined for the effects of EGF on cell growth and the results are shown in Table VII. The effects of TGF-α on growth were also investigated in HMEC from 2 of the carcinomas where sufficient cells were obtained. These results are shown in Figure 9. In all cases examined, EGF and TGF-α significantly ($p < 0.05$) stimulated growth. The degree of stimulation by EGF ranged from 150% to 2912% (average was 1033%) of the controls with no EGF. TGF-α stimulated growth 330% and 639% over controls. In the 2 cases where the effects of EGF and TGF-α were determined, both stimulated growth to the same magnitude. Table VIII presents a summary of the average growth effects of EGF and TGF-α on HMEC from the three different tissue types.

Figure 8 : The effects of EGF and TGF-α on the growth of primary cultures of HMEC from 2 fibroadenomas (FA 74 & 80).

Growth was assessed 14 h after the last medium change, by the incorporation of ^{3}H-TdR (1 μ Ci/ml) into the cells over a 4 h time period. The absolute levels of incorporation have been converted to % of control values, with the level of incorporation in the control condition (0 ng/ml growth factor in the serum-free medium, Appendix 7) being assigned a value of 100 %. Values shown represent the mean ± SEM of triplicate wells of the same condition. EGF and TGF-α at 10 and 100 ng/ml both significantly ($p < 0.05$) stimulated the growth of HMEC from FA 74 (Fig. 8a) and FA 80 (Fig. 8b). In both examples TGF-α at 10 ng/ml stimulated growth to a greater degree than EGF at the same concentration. At 100 ng/ml the stimulation by TGF-α was significantly ($p < 0.05$) reduced relative to level of stimulation by the same growth factor at 10 ng/ml. EGF at 100 ng/ml caused a greater increase in growth than EGF at 10 ng/ml, however, the effect was only significant in cells from FA 74 (Fig. 8a.).

Figure 8a.

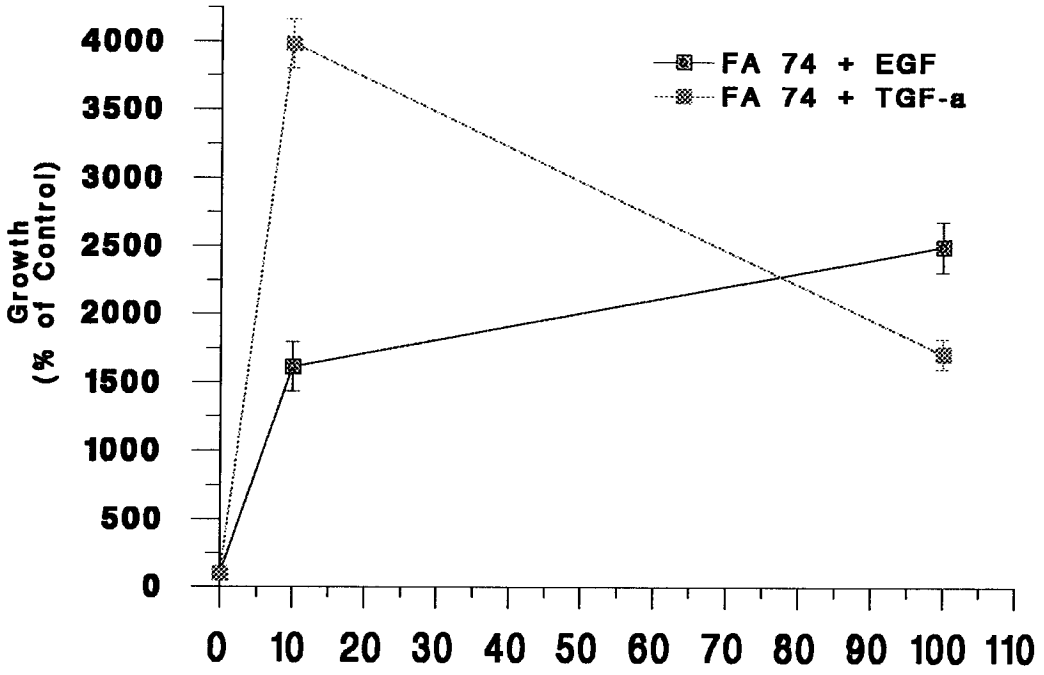


Figure 8b.

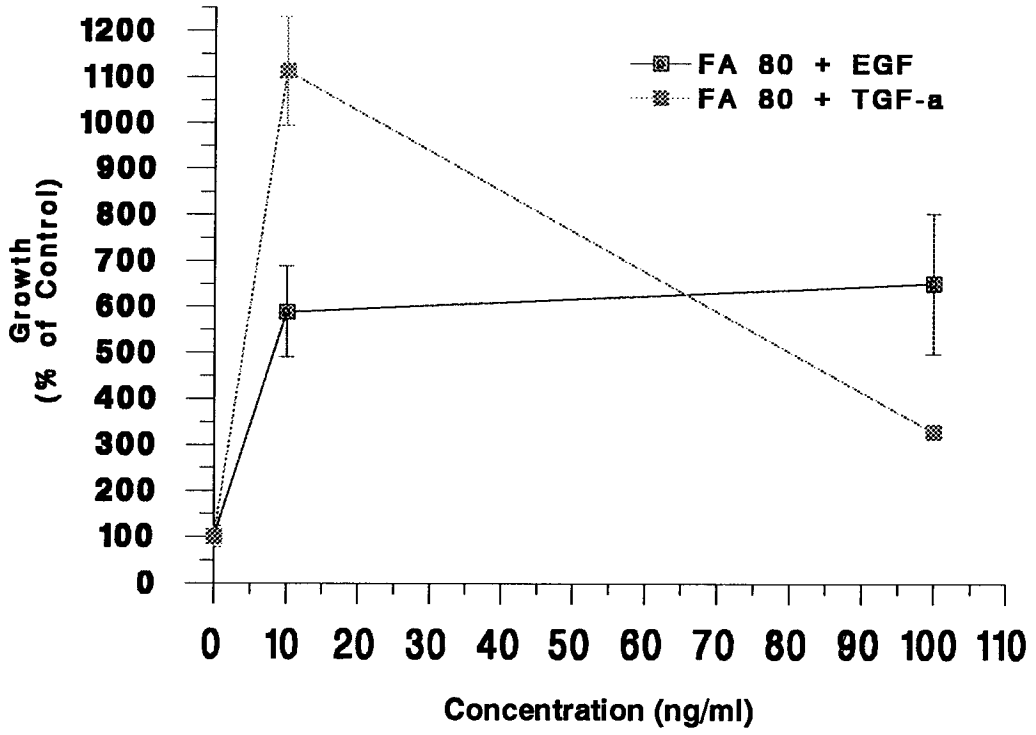


Table VII : The effects of EGF on the growth of primary cultures of HMEC from carcinomas

Specimen no. (div)	Culture conditions	
	Control	+ EGF (10 ng/ml)
HMC 101 (11 div)[a]	100 ± 15[b]	2912 ± 643*
HMC 102 (11 div)	100 ± 39	668 ± 91*
HMC 107 (11 div)	100 ± 58	1582 ± 533*
HMC 108 (13 div)	100 ± 23	150 ± 17*
HMC 109 (12 div)	100 ± 40	1457 ± 403*
HMC 110 (12 div)	100 ± 10	612 ± 20*
HMC 111 (11 div)	100 ± 38	1014 ± 130*
HMC 140 (11 div)	100 ± 14	403 ± 16*
HMC 141 (13 div)	100 ± 13	502 ± 168*
Summary	**100 ± 28 (n=9)[c]**	**1033 ± 224 (n=9)**

a : div = days *in vitro*
b : Values shown represent a percentage of growth relative to the control (control = 0 added growth factor). Values are expressed as the mean ± SEM of each condition done in triplicate
c : n = number of individual samples examined in each condition
* significant stimulation of growth ($p < 0.05$)

Figure 9 : The effects of EGF and TGF-α on the growth of primary cultures of HMEC from 2 fibroadenomas (FA 74 & 80).

Growth was assessed 14 h after the first medium change, by the incorporation of ^{3}H-TdR (1 μCi/ml) into the cells over a 4 h time period. The absolute levels of incorporation have been converted to % of control values, with the level of incorporation in the control condition (0 ng/ml growth factor in the serum-free medium, Appendix 7) being assigned a value of 100 %. Values shown represent the mean ± SEM of triplicate wells of the same condition. EGF and TGF-α at 10 ng/ml both significantly ($p < 0.05$) stimulated the growth of HMEC from HMC 140 and 141. There was no difference in the degree of growth stimulation by EGF and TGF-α.

Figure 9.

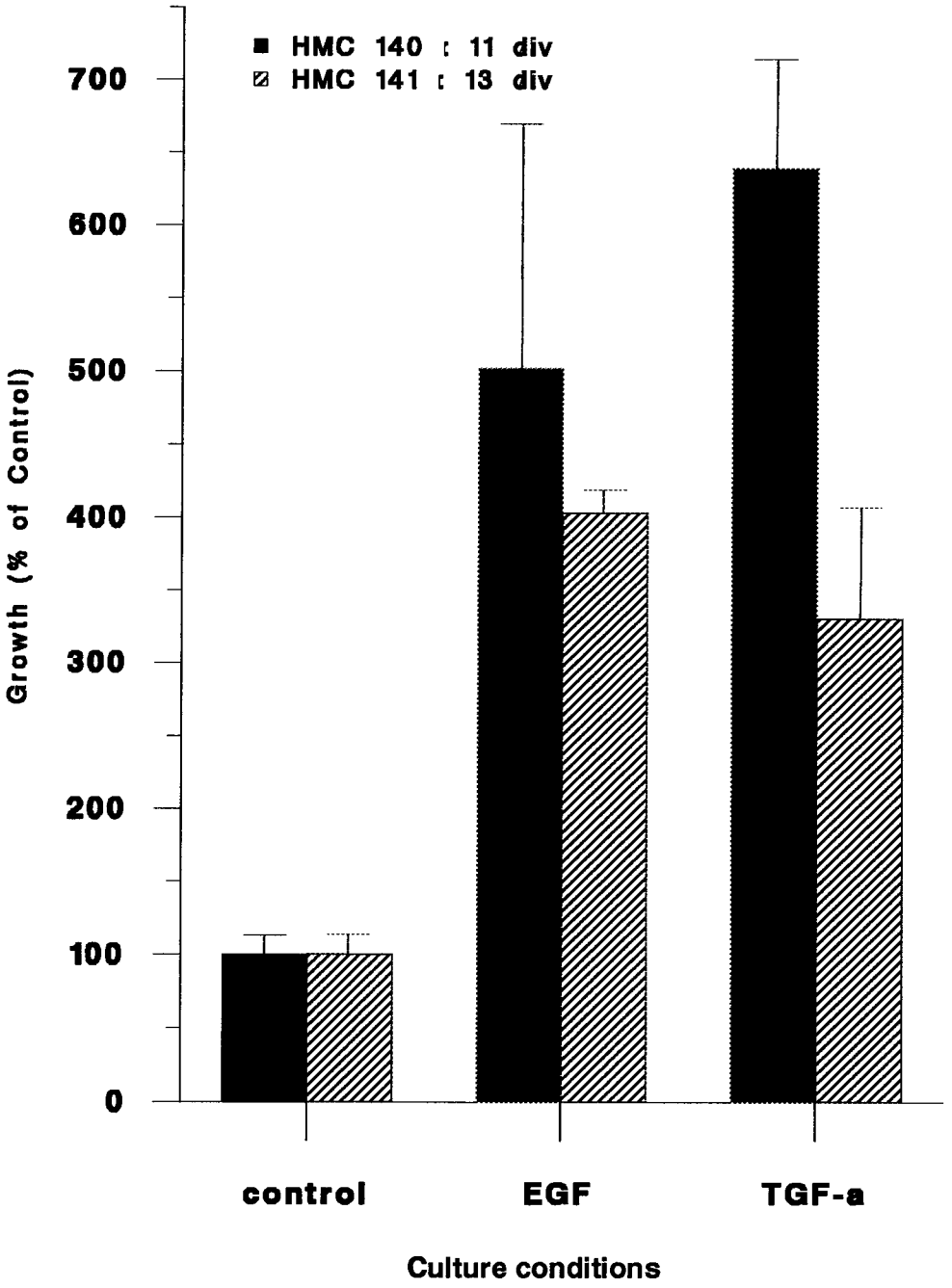

HMC 140 : 11 div
HMC 141 : 13 div
700
600
500
400
300
200
100
0
Growth (% of Control)
control
EGF
TGF-a
Culture conditions

Table VIII : Summary of the effects of EGF and TGF-α on the growth of primary cultures of HMEC from reduction mammoplasties, fibroadenomas and carcinomas

Tissue Type	Average percentage of growth stimulation		
	Control	+ EGF [10 ng/ml]	+ TGF-α [10 ng/ml]
Reduction Mammoplasties	100 ± 12[a] (n=11)[b]	894 ± 97 (n=3)*	N.D.[c]
Fibroadenomas	100 ± 17 (n=19)	923 ± 159 (n=12)*	1088 ± 63 (n=6)*
Carcinomas	100 ± 24 (n=24)	1033 ± 224 (n=12)*	484 ± 168 (n=2)*

a : Values are the averages of individual experiments, and are expressed as the mean ± SEM of each tissue type
b : n = number of individual samples examined in each condition
c : N.D. = no data for these conditions due to limitations in cell numbers from various samples
* significant stimulation of growth compared to control with 0 ng/ml growth factor ($p < 0.05$)

2d. The Effects of a Monoclonal Antibody to EGFR on Growth Stimulation by EGF

Two experiments utilized HMEC from fibroadenomas to investigate the effects of EGF and TGF-α immediately following the first medium change. The rational for these experiments was to determine if these growth factors were able to stimulate the growth of HMEC after only 1 day of exposure to them. These findings were relevant in planning experiments investigating the effects of a monoclonal antibody (MAb) against the EGFR on the growth of cells incubated with EGF. It would be ideal to investigate the effects of the MAb after a minimal amount of time *in vitro* in order to minimize the amount of antibody required for each experiment. The results of these experiments are shown in Figure 10. Sixteen hours after the addition of EGF or TGF-α, growth was only significantly ($p < 0.05$) stimulated by EGF in HMEC from FA 78.

The effects of MAb 528, a monoclonal antibody which competitively blocks EGF binding to EGFR, were examined in cultures of HMEC from 2 fibroadenomas and the results shown in Figures 11a and 11b. Since these growth factors do not significantly stimulate growth after only 1 medium change (Figure 10), the medium in these experiments was changed every other day for 6 days. Fresh growth factor and antibody were added with each change. The MAb reduced the degree of growth stimulation in both cases examined, however, the reduction in stimulation was statistically significant ($p < 0.05$) for HMEC from only one of the fibroadenomas (FA 81, Figure 11a.).

Figure 10 : The effects of EGF and TGF-α on the growth of primary cultures of HMEC from 2 mammary carcinomas (HMC 140 & HMC 141).

Growth was assessed 14 h after the last medium change, by the incorporation of ^{3}H-TdR (1 μCi/ml) into the cells over a 4 h time period. The absolute levels of incorporation have been converted to % of control values, with the level of incorporation in the control condition (0 ng/ml growth factor in the serum-free medium, Appendix 7) being assigned a value of 100 %. Values shown represent the mean ± SEM of triplicate wells of the same condition. EGF caused a significant ($p < 0.05$) stimulation of growth in HMEC from FA 78. None of the other treatments caused any significant effects on the growth of HMEC from either FA 77 or FA 78.

Figure 10.

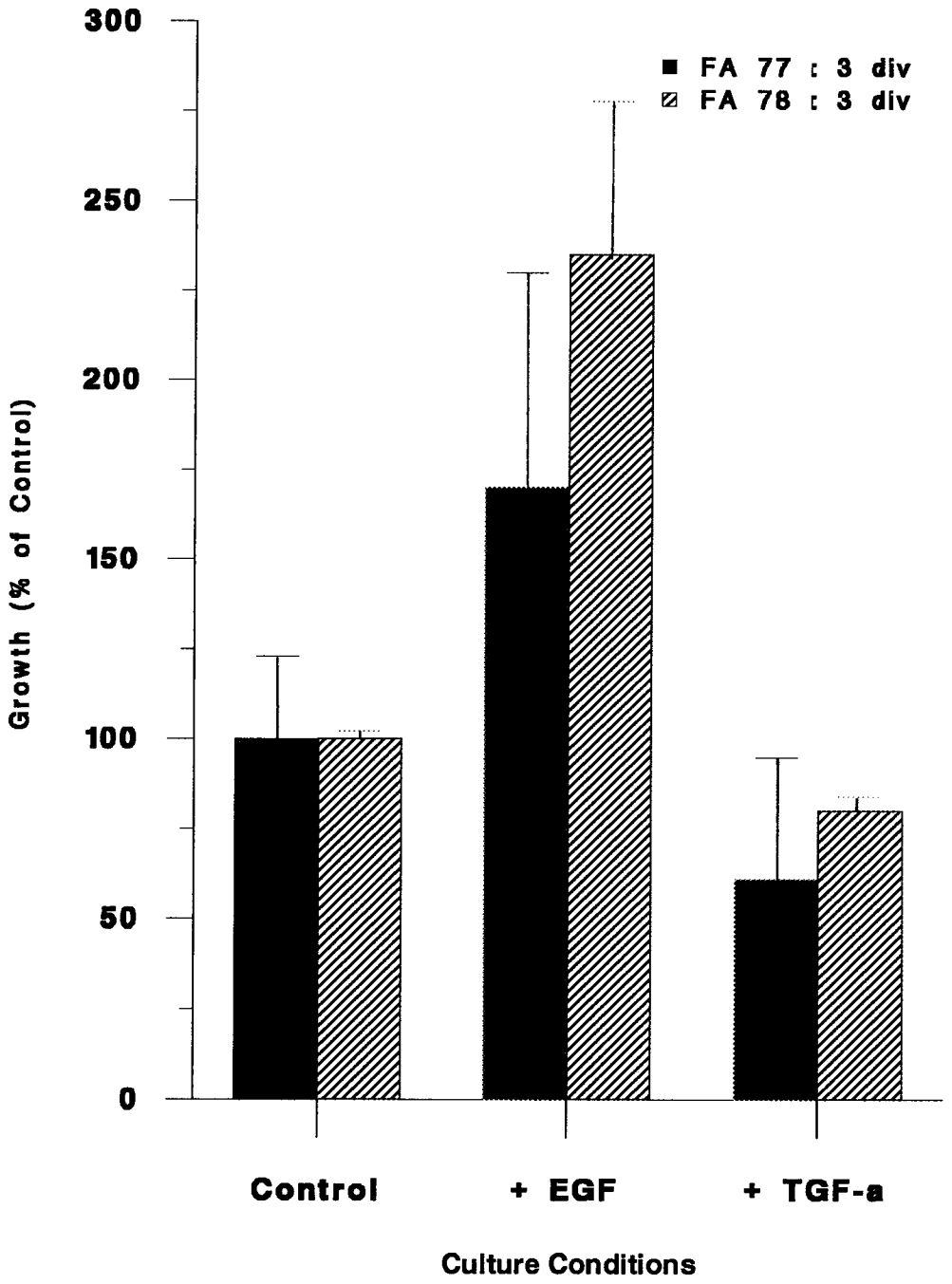

300
250
200
150
100
50
0
Growth (% of Control)
FA 77 : 3 div
FA 78 : 3 div
Control
+ EGF
+ TGF-a
Culture Conditions

Figure 11 : The effect of MAb 528 on growth stimulation by EGF.

The effects of MAb 528, which competitively blocks EGF binding to EGFR, and EGF at on the growth of primary cultures of HMEC isolated from 2 fibroadenoma samples (FA 81, Fig.11a., FA 82, Fig.12a.) was examined. Growth was assessed by the incorporation of ^{3}H-TdR (1 μCi/ml) into the cells over a 4 h time period. The absolute levels of incorporation have been converted to % of control values, with the level of incorporation in the control condition (0 ng/ml EGF) being assigned a value of 100 %. Values shown represent the mean ± SEM of triplicate wells of the same condition. EGF caused a significant ($p < 0.05$) stimulation of growth in both FA 81 and FA 82. MAb 528 reduced the degree of stimulation by EGF in both cases, however, this effect was statistically significant only in FA 81 (Fig.11a.).

Figure 11a.

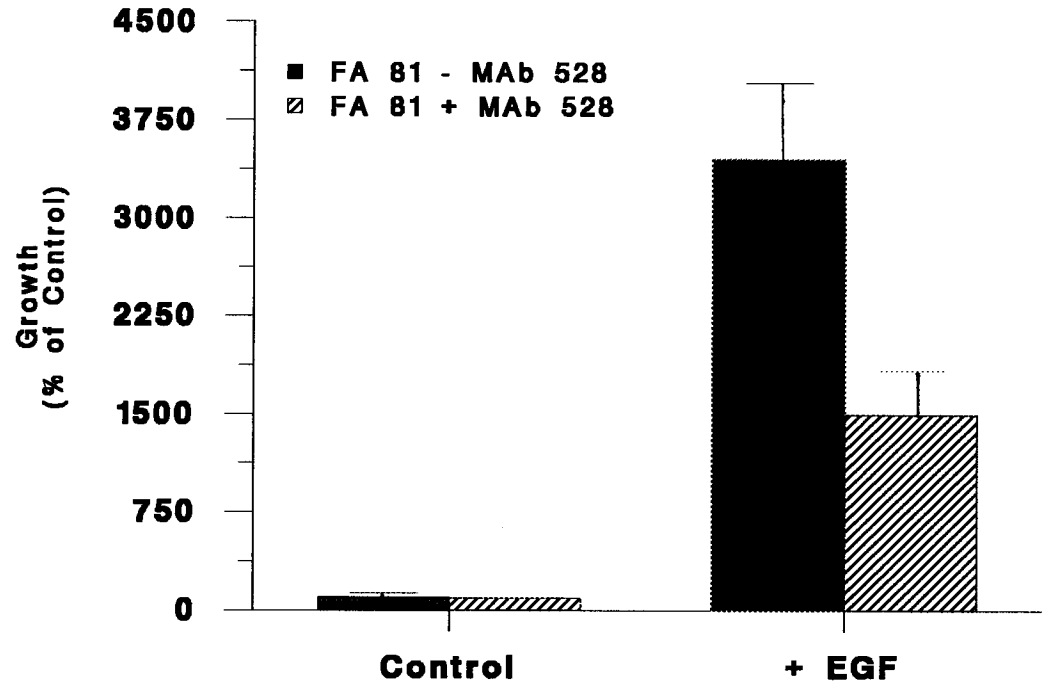
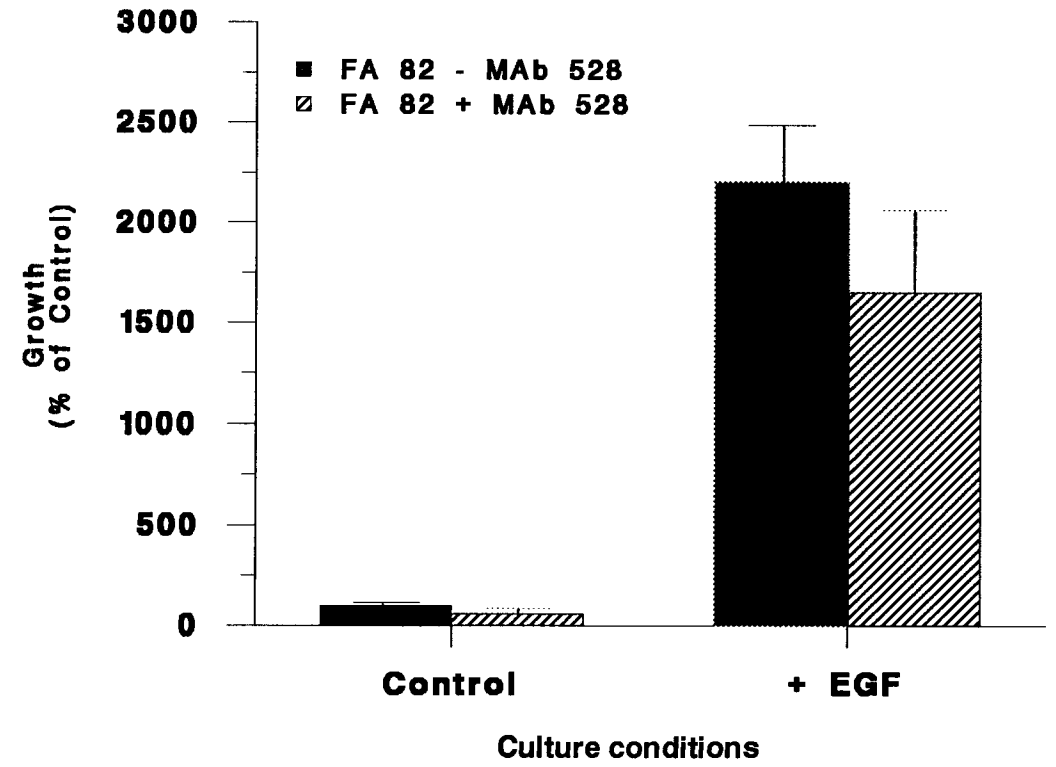


Figure 11b.



3 : The Combined Effects of E_2 and EGF on the Growth of Primary Cultures of HMEC From Reduction Mammoplasties, Fibroadenomas and Carcinomas

The effects of E_2 and EGF added in combination were examined in primary cultures of HMEC from 1 reduction mammoplasty, 6 fibroadenomas and 2 carcinomas. The results of these experiments are shown in Table IX. There was no additive or synergistic effect of E_2 and EGF in combination in HMEC from normal tissue; that is, the growth effect of E_2 and EGF together was the same as the stimulation observed in response to EGF alone. In 2 of the 6 cultures of HMEC from fibroadenomas, a significant ($p < 0.05$) synergistic effect on the stimulation of growth by the addition of E_2 and EGF in combination was observed. The results of 1 experiment are illustrated in Figure 12. A synergistic effect of E_2 plus EGF was also observed in HMEC from 1 of the 2 carcinomas and the results of this experiment are illustrated in Figure 13.

Prior to the initiation of these experiments measuring growth responses of HMEC to E_2 and EGF, a number of experiments were attempted to determine the effects of these same treatments on gene expression. Though these experiments were unsuccessful, in one experiment sufficient cells were grown to yield cell weight measurements for each of the experimental conditions. These results are presented in Figure 14. Each condition consisted of only one culture dish, therefore, it is not possible to determine the statistical error in these measurements. However, these results are not presented as statistically significant, but rather as supportive evidence of a synergistic effect of E_2 plus EGF on the growth of HMEC from a fibroadenoma.

Table IX : The effects of 17β-estradiol (E_2) plus EGF on the growth of primary cultures of HMEC from a reduction mammoplasty, fibroadenomas and carcinomas

Specimen no. (div)[a]	Culture Conditions			
	Control	+ E_2 (10 nM)	+ EGF (10 ng/ml)	+ E_2 (10 nM) + EGF (10 ng/ml)
Redn 12 (8 div)	100 ± 22[b]	80 ± 26	264 ± 30*	292 ± 12*
FA 43 (8 div)	100 ± 17	146 ± 26	167 ± 43*	167 ± 30*
FA 45 (8 div)	100 ± 12	120 ± 10	197 ± 15*	201 ± 23*
FA 51 (11 div)	100 ± 9	129 ± 17	1047 ± 283*	1643 ± 245**
FA 54 (9 div)	100 ± 11	81 ± 30	753 ± 161*	1866 ± 147**
FA 81 (7 div)	100 ± 28	110 ± 25	3455 ± 583*	3249 ± 138*
FA 82 (7 div)	100 ± 17	74 ± 6	2203 ± 284*	1917 ± 331*
HMC 108 (13 div)	100 ± 23	57 ± 21	150 ± 17*	200 ± 39*
HMC 111 (13 div)	100 ± 40	112 ± 19	1457 ± 403*	3195 ± 795**

a : div = days *in vitro*

b : Values shown represent a percentage of growth relative to the control (control = serum-free medium with no added E_2 or EGF, Appendix 7). Values are expressed as the mean ± SEM of each condition done in triplicate

* significant ($p < 0.05$) stimulation of growth compared to the control

** significant ($p < 0.05$) stimulation of growth compared to the plus EGF condition

Figure 12 : The effect of E_2 plus EGF on the growth of primary cultures of HMEC from 2 fibroadenomas (Fig. 12a., FA 51, Fig. 12b, FA 54).

Growth was assessed by the incorporation of ^{3}H-TdR (1 μCi/ml) into the cells over a 4 h time period. The absolute levels of incorporation have been converted to % of control values, with the level of incorporation in the control condition (0 nM E_2 and 0 ng/ml EGF) being assigned a value of 100 %. Values shown represent the mean ± SEM of triplicate wells of the same condition. E_2 alone caused no significant effect on growth in either FA 51 or FA 54. EGF caused a significant ($p < 0.05$) stimulation of growth in both FA 51 and FA 54. E_2 and EGF in combination caused a significantly greater ($p < 0.05$) increase in growth over EGF alone in both samples.

Figure 12a.

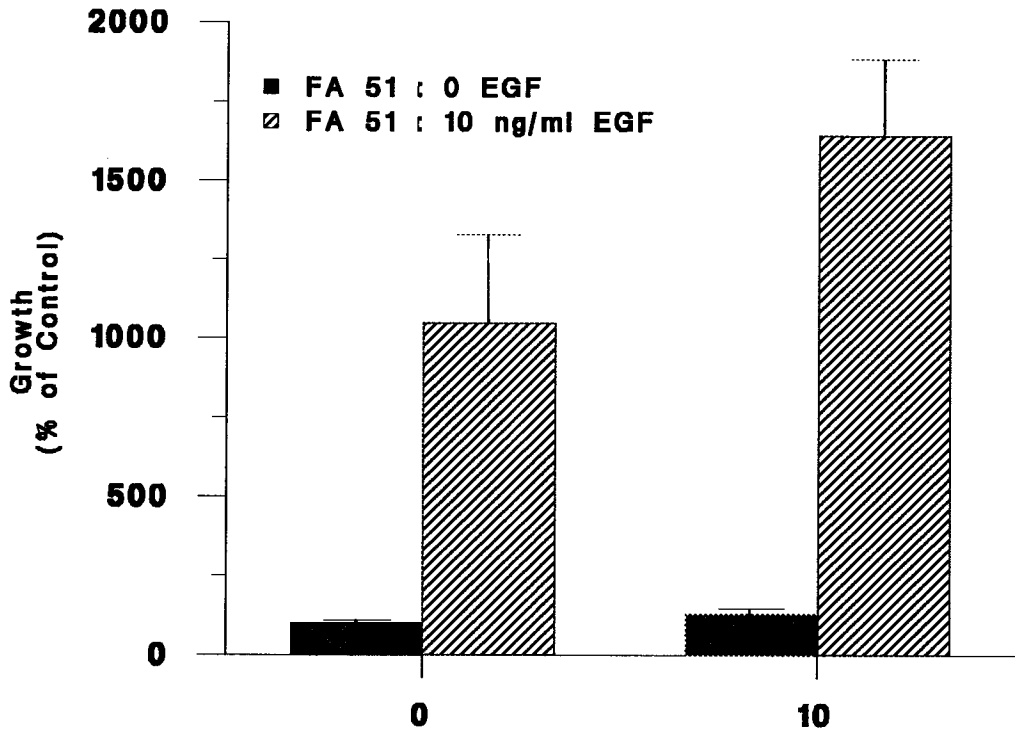


Figure 12b.

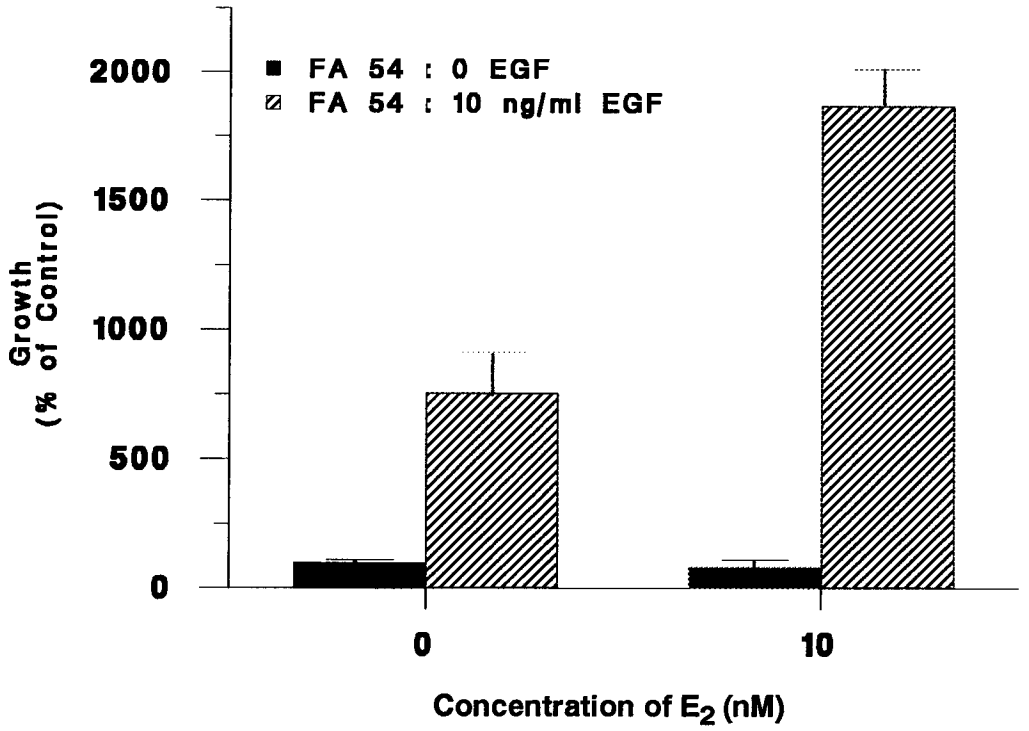


Figure 13 : The effects of E_2 plus EGF on the growth of primary cultures of HMEC from a mammary carcinoma (HMC 111).

Growth was assessed by the incorporation of ^{3}H-TdR (1 μCi/ml) into the cells over a 4 h time period. The absolute levels of incorporation have been converted to % of control values, with the level of incorporation in the control condition (0 nM E_2 and 0 ng/ml EGF) being assigned a value of 100 %. Values shown represent the mean ± SEM of triplicate wells of the same condition. E_2 alone caused no significant effect on the growth of HMC 111. EGF caused a significant ($p < 0.05$) stimulation of growth. E_2 and EGF in combination caused a significantly greater ($p < 0.05$) increase in growth than EGF alone.

Figure 13.

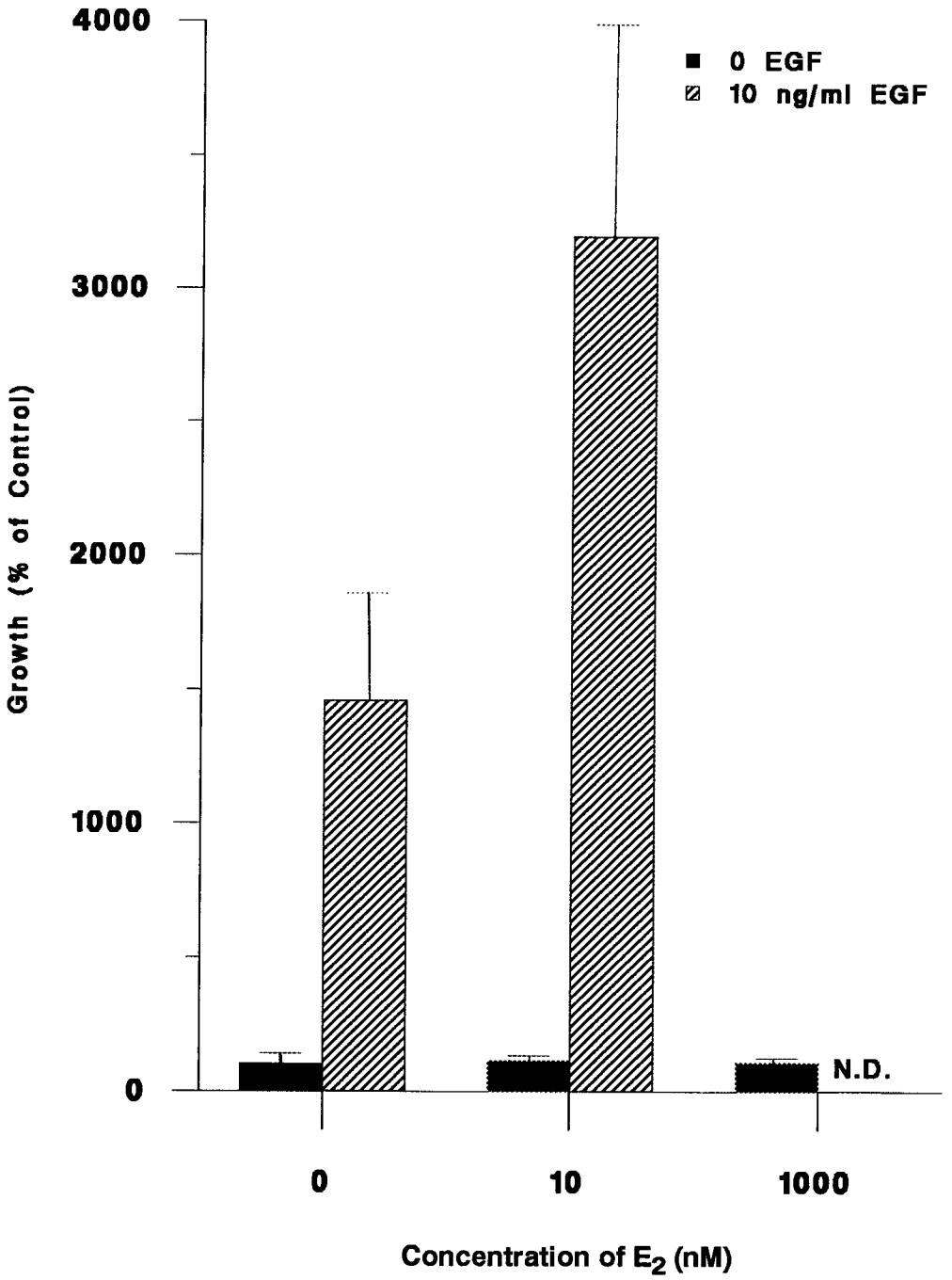
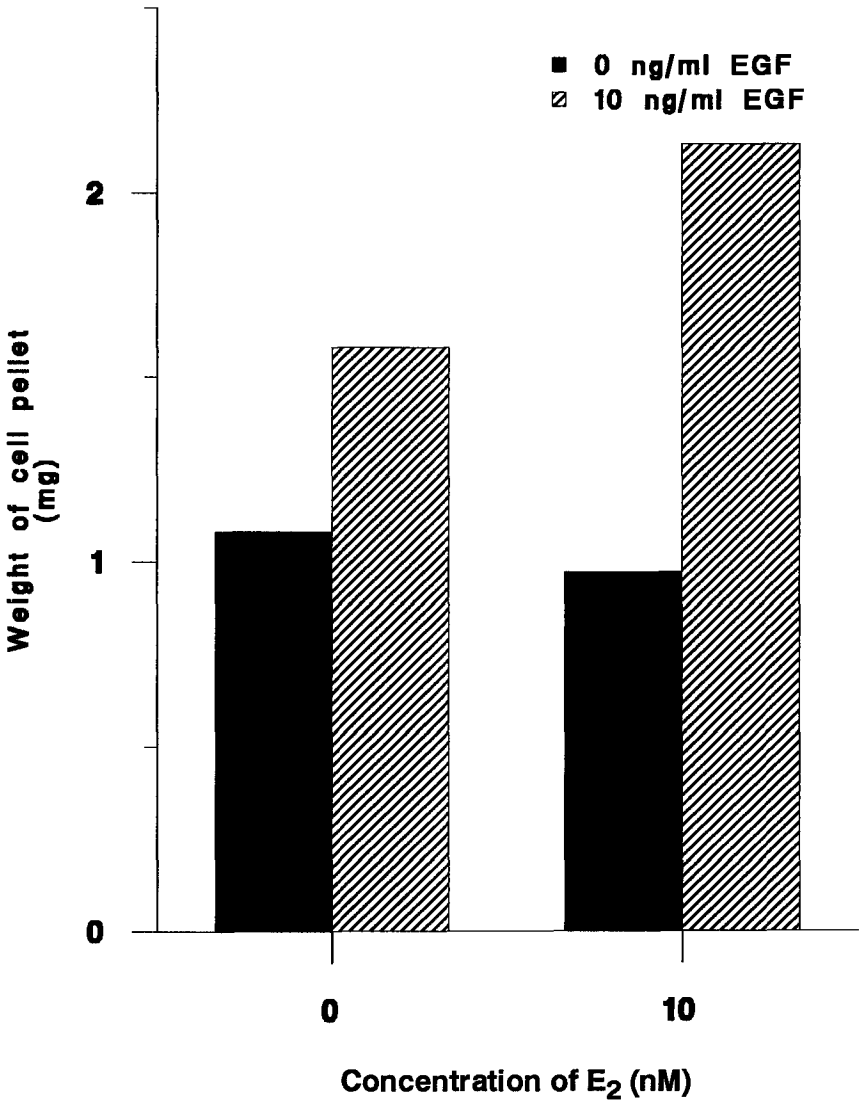

4000
3000
2000
1000
0
Growth (% of Control)
0 EGF
10 ng/ml EGF
N.D.
0
10
1000
Concentration of E_2 (nM)

Figure 14 : The effects of E_2 plus EGF on the growth of primary cultures of HMEC from a fibroadenoma (FA 41).

Cells were harvested from the dishes by mild trypsinization and the cell suspension was pelleted by centrifugation. Cell pellets were then weighed. Estrogen at 10 nM caused a 10% reduction in cell weight. EGF at 10 ng/ml caused a 46% increase in cell weight. Estrogen and EGF in combination caused a 100% increase in cell weight.

Figure 14.


0 ng/ml EGF
10 ng/ml EGF
Weight of cell pellet (mg)
2
1
0
0
10
Concentration of E_2 (nM)

4 : The Effect of E_2 and EGF on the Growth of Primary Cultures of HMEC Cultured on Feeder Layers of Mitomycin-C Treated Fibroblasts

Since E_2 does not appear to have a direct effect on the growth of HMEC , the effects of E_2 may be mediated by production of paracrine growth factors by fibroblasts or may require direct contact of HMEC with mammary fibroblasts. For these reasons, three preliminary studies examined the effects of E_2 and/or EGF on the growth of HMEC grown on mitomycin-C treated fibroblasts feeder layers. A dose response curve for mitomycin-C demonstrated that 1.0 - 10.0 μg/ml mitomycin-C rendered the fibroblasts incapable of growth. However, the higher concentrations of mitomycin-C (5.0 and 10.0 μg/ml) were observed to cause cell detachment and death. For this reason fibroblasts were treated with 1 μg/ml of mitomycin-C. After mitomycin-C treatment, the fibroblasts remained in serum-free medium for a further 24 h to remove traces of serum or mitomycin-C. The results of these studies are presented in Figures 15 and 16. E_2 at 1000 nM significantly ($p < 0.05$) inhibited the growth of HMEC from FA 87 grown on mitomycin-C treated fibroblasts (Figure 15). EGF was observed to stimulate the growth of FA 87 grown on fibroblasts. The growth of HMEC from HMC 130 on collagen-coated dishes was significantly inhibited by 1000 nM E_2 (Figure 16). In contrast to this E_2 had no effects on the growth of HMEC from the same sample grown on fibroblasts. HMC 129 grown on either collagen or fibroblasts did not respond to E_2 (Figure 16).

Figure 15 : The effect of E_2 and EGF on the growth of primary cultures of HMEC from a fibroadenoma (FA 87 : 11 div).

Cells were grown on a monolayer of mitomycin-C treated fibroblasts. Growth was assessed by the incorporation of ^{3}H-TdR (1 μCi/ml) into the cultures over a 4 h period. The absolute levels of incorporation have been converted to % of control values, with the level of incorporation in the control condition (0 nM E_2 & 0 ng/ml EGF) being assigned a value of 100 %. Values shown represent the mean ± SEM of triplicate wells of the same condition. E_2 at 1000 nM caused a significant inhibition ($p < 0.05$) of growth in FA 87. EGF at 10 ng/ml caused a significant stimulation ($p < 0.05$) of growth.

Figure 15

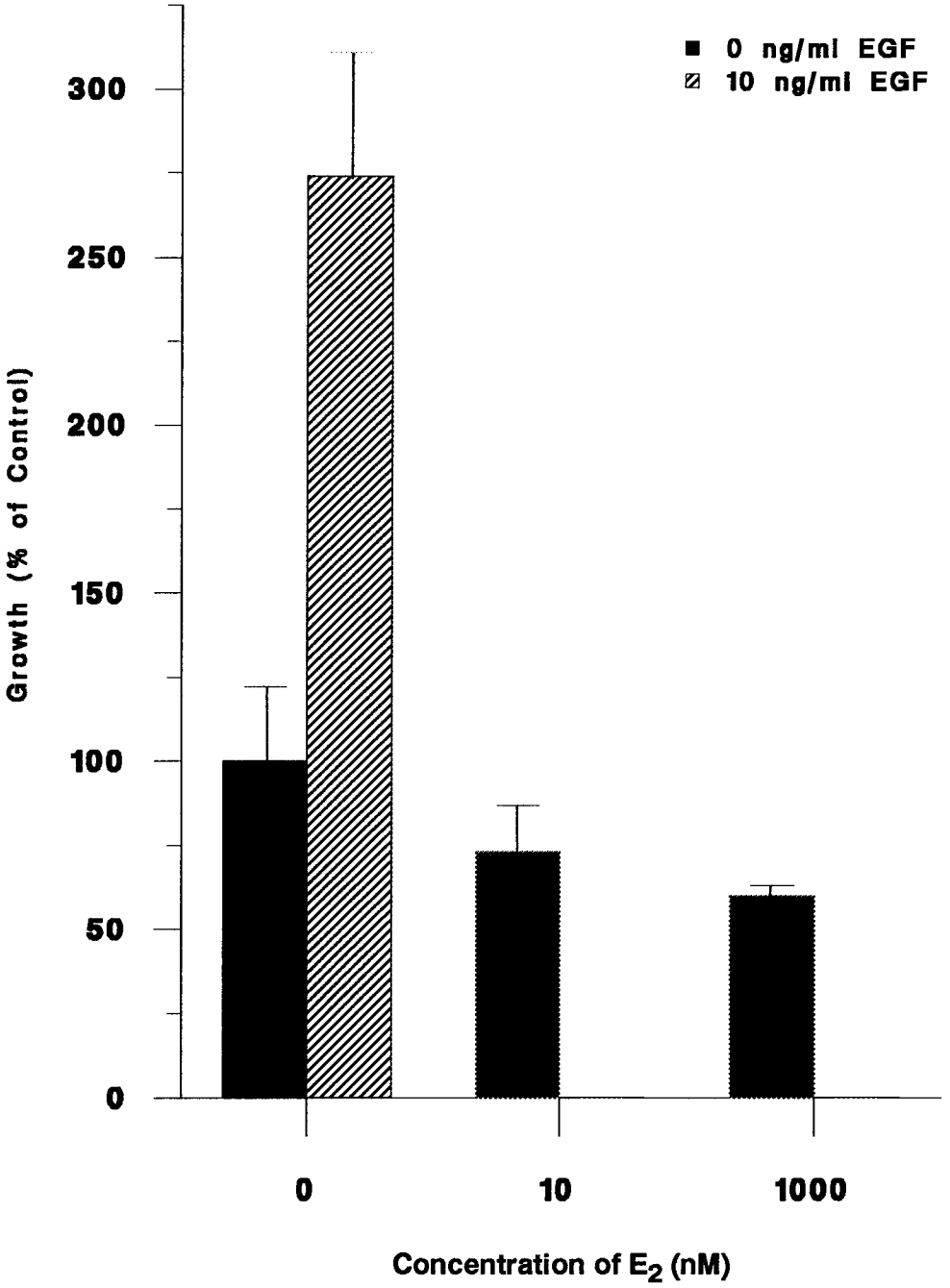
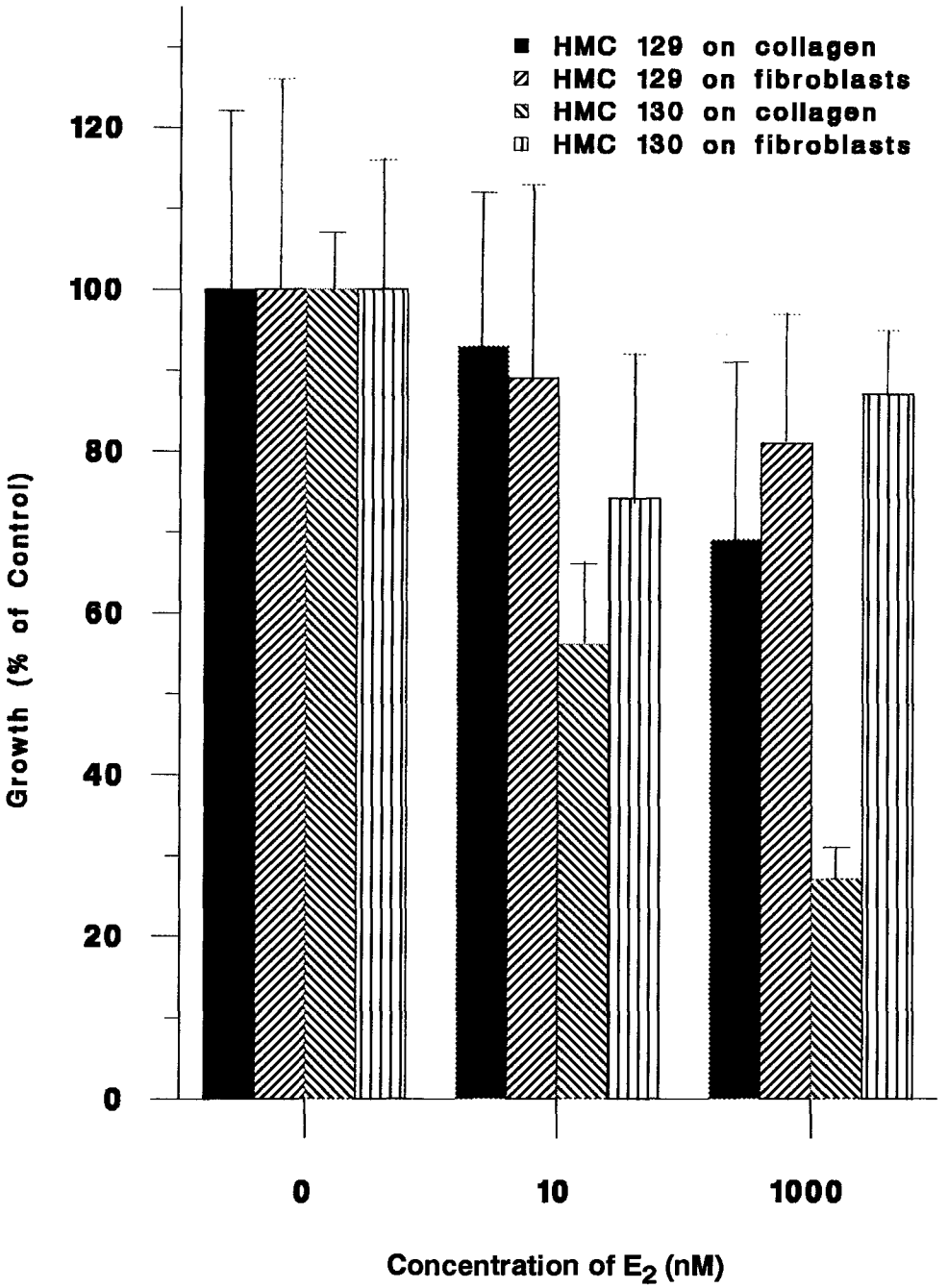

0 ng/ml EGF
10 ng/ml EGF
Growth (% of Control)
300
250
200
150
100
50
0
0
10
1000
Concentration of E_2 (nM)

Figure 16 : The effect of E_2 on the growth of primary cultures of HMEC from 2 carcinomas (HMC 129 and HMC 130).

Cells were grown on either dehydrated collagen or a monolayer of mitomycin-C treated fibroblasts. Growth was assessed by the incorporation of ^{3}H-TdR (μCi/ml) into the cultures over a 4 h period. The absolute levels of incorporation have been converted to % of control values, with the level of incorporation in the control condition (0 nM E_2) being assigned a value of 100 %. Values shown represent the mean ± SEM of wells of the same condition. E_2 at 1000 nM caused a significant inhibition ($p < 0.05$) of growth in HMC 130 grown on collagen, however in none of the other conditions was any significant effect of estrogen observed.

Figure 16


HMC 129 on collagen
HMC 129 on fibroblasts
HMC 130 on collagen
HMC 130 on fibroblasts
120
100
80
60
40
20
0
Growth (% of Control)
0
10
1000
Concentration of E_2 (nM)

CHAPTER 4

DISCUSSION

1. The Effects of E_2 on the Growth of HMEC

Our studies as well as those of others have shown that E_2 at physiological concentrations can stimulate the growth of HMEC in primary cultures in the presence of serum [Emerman et al., 1990; Mauvais-Jarvis et al., 1986; Calaf et al., 1986b; Longman and Beuhring, 1987], however, we were unable to demonstrate a stimulatory effect on the growth of cells from normal, benign and malignant mammary gland tissues in the serum-free medium used in the experiments described in this thesis (Appendix 7). This is consistent with previously published reports showing no effect of E_2 on the growth of human or rodent MEC in serum-free primary culture [Yang et al., 1982; Imagawa et al., 1985; Hahm and Ip, 1990].

In the carcinoma samples, there was no growth response to E_2 in spite of the fact that all samples were ER^+. Unfortunately, ER levels were not determined for the normal and fibroadenoma samples. However, both of these tissue types have been shown to contain significant numbers of ER^+ cells in a majority of specimens [Petersen et al., 1987; Giri et al., 1989]. Furthermore, it has been shown that ER^+ cells from normal samples and fibroadenomas cultured under similar conditions to ours remain ER^+ throughout the culture period [Balakrishnan et al., 1987; Malet et al., 1991]. Therefore, it is unlikely that the absence of ER^+ cells in our samples is a factor related to the absence of estrogen-stimulated growth. We are planning to measure the levels of ER in the original tissues and to study the effects of our cell-culture conditions on ER levels using the immunohistochemical ER detection kit produced by Abbot laboratories [Malet et al., 1991]. Assuming that the absence of an estrogenic stimulation of growth in serum-free medium is not due to a lack of ER, the data

suggest that either factors present in the medium block or mask an estrogenic stimulation of growth or, alternatively, that factors absent from the medium are required for an E_2 effect.

We examined the possibility that the pharmacological concentration of insulin in our serum-free-medium (10 μg/ml) was masking an estrogenic effect on cell growth, as has been demonstrated for the MCF-7 breast cancer cell line [van der Burg et al., 1988; Ruedl et al., 1990]. A 100-fold reduction of the initial insulin concentration caused a significant reduction in cell growth in all of the cases examined (Table V). Differences in the growth response of the cells to E_2 were observed in the low-insulin medium. E_2 failed to inhibit growth in the low insulin medium and there was a E_2 stimulation of growth observed in a fibroadenoma (Fig. 6.). These experiments suggest that insulin is indeed blocking E_2 growth-stimulating effects. It is possible that high insulin concentrations block a growth regulatory pathway normally utilized in E_2 stimulation of growth.

The role of insulin in the development of alveolar structures, or simply for maintenance of mammary epithelial cells *in vitro,* is unclear. Early studies showed that injections of E_2 and progesterone into diabetic (no significant insulin present) male rabbits induced the formation of extensive lobuloalveolar structures in mammary gland tissue [Norgren et al., 1968]. Many studies have shown that mammary epithelial cells in serum-free medium are stimulated to replicate by the addition of insulin [Stockdale and Topper, 1966; Wang and Amor, 1971], but changes in the cells' response to insulin during the time *in vitro* have led to debate over the physiological relevance of insulin to mammary gland tissue [Friedberg et al., 1970]. Friedberg's group observed that mammary epithelial cells on day 1 *in vitro* were insensitive to insulin, but after day 2 *in vitro* the cells acquired insulin sensitivity. Although this finding suggests that insulin sensitivity *in vitro* is an acquired effect, it is equally possible that the trauma to the tissue during transplant procedures or culturing the tissue temporarily renders the mammary epithelial tissue incapable of responding to insulin.

Although insulin does not appear to be required for normal mammary gland development *in vivo*, the large number of studies showing a variety of effects of insulin on mammary epithelial cells *in vitro* suggest a role for insulin in mammary gland development. Insulin added to serum-free explant cultures of mammary glands causes extension of cell viability and stimulation of DNA synthesis [Oka et al., 1974]. The *in vitro* studies of insulin effects on MEC also support a role for insulin in lactational activity. The presence of insulin is required in combination with prolactin and a glucocorticoid to induce terminal differentiation (milk product production) in mammary explant cultures of hormonally-primed virgin mice [Oka et al., 1974]. Furthermore, addition of insulin to the medium of MEC cultures stimulates an increase in the amount of rough endoplasmic reticulum, an increase in the number of Golgi complexes, milk protein production and lactose synthetase activity [Mills and Topper, 1970; Emerman et al., 1977; Emerman and Pitelka, 1977; Katiyar et al., 1978; Vonderhaar, 1977].

It has been demonstrated that insulin at 10 μg/ml interacts with the insulin-like growth factor 1 (IGF-1) receptor [Rechler et al., 1986]. Therefore, studies demonstrating the requirement of high concentrations of insulin for significant growth of serum-free cultures of MEC may have actually been demonstrating a requirement for IGF-1 [Ethier et al., 1987, Deeks et al., 1988]. Addition of IGF-1 to serum-free cultures of E_2-dependent breast cancer cell lines has been found to be growth stimulatory [Karey and Sirbasku, 1988], and it will be important to determine if lower concentrations of IGF-1 are able to replace the high concentrations of insulin used in our serum-free cultures. The importance of the IGF-1 receptor in E_2-responsive growth is implicated by the finding that E_2 stimulation of growth in human breast cancer cell lines is accompanied by increased production of IGF-1 [Huff et al., 1986]. It is possible that in E_2 stimulation of growth in HMEC may involve production of growth factors (eg. IGF-1) that interact with the IGF-1 receptor, but this interaction is blocked in the presence of high concentrations of insulin due to insulin occupation of the IGF-1 receptor [van der Burg et al., 1990].

Using the same serum-free medium used in the experiments of this thesis, but without hydrocortisone, Yang and coworkers fail to show any growth effect by E_2 [Yang et al., 1982]. Although their study examined only two normal specimens and one cancerous sample, the experiments suggest that hydrocortisone is not blocking or masking a growth effect of E_2. However, these experiments utilized the high concentration of insulin, which could still block or mask an E_2 effect on growth, even in the absence of hydrocortisone. Clearly a more detailed study is required to conclude that hydrocortisone does not modulate E_2 responses *in vitro*.

The effects of cholera toxin in the serum-free medium on E_2 regulation of growth are also not known. The addition of cholera toxin to the medium is based on the finding that it selectively stimulates the growth of the MEC, whereas mammary stromal cells do not grow in its presence [Taylor-Papadimitriou et al., 1980]. The growth stimulatory effects of cholera toxin on MEC are thought to be due to increased intracellular levels of cAMP, as analogues of cAMP are also able to stimulate the growth of MEC. No studies have been done to show how cholera toxin may modulate the growth effects of E_2 or other mammotrophic hormones *in vitro*. Our lab is currently investigating the effects of significantly reducing or eliminating cholera toxin from the serum-free medium in order to eliminate the problem of uncharacterized effects of cholera-toxin. It will be interesting to compare the growth effects of E_2 and other hormones in medium without cholera toxin present. It is possible that via an increase in intracellular cAMP levels, cholera toxin could mask growth modulatory effects of any hormones or growth factors also operating through a cAMP second messenger system.

In summary, the medium components required to support growth in serum-free primary culture are quite likely responsible, at least in part, for the finding that E_2 is unable to stimulate the proliferation of HMEC from any of the samples examined (with the notable exception of one fibroadenoma in the low insulin medium). However, the finding that addition of EGF to the medium is consistently able to stimulate the proliferation of HMEC from normal, benign and

malignant tissues demonstrates that there is no factor in the medium which renders HMEC incapable of growing at increased rates. The growth stimulation by EGF also shows that the absence of stimulation by E_2 is not due to the fact that the cells have already reached a maximal growth rate.

As already discussed, E_2 stimulation of HMEC growth in serum containing media has previously been demonstrated by us [Emerman et al., 1990] and others [Mauvais-Jarvis et al., 1986; Calaf et al., 1986b; Longman and Beuhring, 1987]. The E_2 stimulation of growth that we reported was observed in medium containing dextran charcoal-treated serum. This treatment removes steroid hormones present in the serum, but does not remove peptide growth factors. However, if the serum is further chemically treated to break disulfide bridges, and therefore inactivate peptide growth factors, serum is no longer able to support E_2 stimulation of proliferation to the same magnitude [Ruedl et al., 1990]. This finding suggests that there is one or more peptide factors present in serum which are required for E_2 stimulation of growth *in vitro*; it is likely that the significant levels of EGF in serum are involved in this effect (discussed in more detail later in the Discussion).

The absence of other factors in the serum-free culture system used in these experiments may also be related to the absence of growth stimulation by E_2. One factor that is very different *in vitro* than *in vivo* and is directly involved in modulating both growth and differentiation responses to hormones is the extracellular substrate to which the HMEC adheres [Blum et al., 1989]. The ability of a biological substrate to modulate MEC differentiation *in vitro* was clearly demonstrated when Emerman and coworkers cultured normal mouse MEC on floating collagen Type I gels [Emerman et al., 1977; Emerman and Pitelka, 1977]. Previously, cytodifferentiation of these cells *in vitro* had not been possible. However, when grown on floating collagen gels the MEC could be induced to undergo cytodifferentiation. Hormonal regulation of casein synthesis and secretion was also demonstrated. In a

modification of the technique developed by Emerman et al., Yang and co-workers cultured MEC inside of 3-dimensional collagen Type I gels. In their system, MEC was shown to both proliferate and differentiate into duct-like structures [Yang et al. 1980 and 1982]. Although a collagen Type I substrate provides a sufficient substrate for cell growth and cytodifferentiation, it may not be sufficiently able to mimic the *in vivo* situation to allow E_2 stimulation of growth.

Co-culture studies involving both fibroblasts and/or adipocytes grown together with epithelial cells provide an *in vitro* condition more like the *in vivo* setting. When mouse MEC are cultured in 5 % serum on irradiated adipocytes (3T3-L1), the growth level is greater than that of cells grown on non-adipocyte 3T3 cells [Levine and Stockdale, 1984]. The effect of the adipocytes is partially due to direct substrate effects, as demonstrated by the observation that cell-free ECM preparations of the adipocytes are able to stimulate the growth of mouse MEC. A role for soluble factors produced by the adipocytes is also shown by the ability of conditioned media from adipocytes to stimulate growth of the mouse MEC. In another paper by the same researchers, adipocytes as a substrate are shown to induce duct-like morphogenesis and the production of a basement membrane [Wiens et al., 1987]. In experiments similar to those of Emerman et al. [1977] and Emerman and Pitelka [1977], these researchers showed that addition of lactogenic hormones to the medium of mouse MEC cultured on 3T3-L1 adipocytes are able to stimulate secretory differentiation, as indicated by morphological criteria [Wiens et al., 1987]. The ability of hormones to stimulate secretory differentiation requires that the adipocytes be alive, in comparison to the stimulation of growth which is observed in cells grown on lethally irradiated adipocytes.

Other studies have examined the ability of mammary fibroblasts to modulate both growth and differentiation responses of MEC to E_2 in co-culture experiments. Haslam's group showed that E_2 stimulation of progesterone receptor synthesis does not require the presence of live fibroblasts [Haslam and Levely, 1985, Haslam, 1986]. Gluteraldehyde treated fibroblasts,

fibroblast conditioned medium or collagen Type I coating of the plates are all sufficient to allow E_2 stimulation of progesterone receptor levels. In contrast to the progesterone receptor stimulation, E_2 stimulation of DNA synthesis requires the presence high numbers of live fibroblasts, or if the number of fibroblasts is reduced, direct contact of the fibroblast and epithelial cells is required. The reason for the apparent discrepancies between the findings of Haslam's group, who showed that live fibroblasts are required for E_2 stimulation of growth, and Wien's group, who showed that lethally irradiated adipocytes, but not live 3T3 fibroblasts, are sufficient is not clear. The most intuitive hypothesis is that the findings in Haslam's laboratory are more relevant as the stromal cells used in those studies were of mammary origin, as opposed to the 3T3 derivatives used in the studies by Levine and Stockdale [1984] and Wiens et al. [1987].

The different *in vitro* requirements for the two different E_2 responses studied by Haslam and co-workers are likely indicative of the existence of at least two separate mechanisms of E_2 action. More specifically, it is likely that E_2 stimulation of progesterone receptors is via a mechanism involving alteration of the extracellular matrix composition. Collagen type I, a substitute for the fibroblasts in E_2 stimulation of progesterone receptors, is a substrate sufficient to allow production of extracellular matrix components by MEC. Cells grown on plastic do not produce a basement membrane and progesterone receptors are not stimulated in MEC grown on plastic. The growth stimulatory effects of E_2 require the presence of live fibroblasts. This observation supports the hypothesis that E_2 stimulation of growth is via production of diffusible paracrine-acting growth factors by E_2-responsive cells. The presence of multiple mechanisms of E_2 action on the parameter of cell growth is also demonstrated by the findings in this thesis that E_2 is able to significantly inhibit the growth of HMEC from all tissue types. This finding demonstrates that growth inhibition by high dose E_2 is not affected by the conditions which prevented E_2 stimulation of growth.

Although preliminary, the results of the experiments in this thesis examining the growth of HMEC grown on mitomycin-C treated normal mammary fibroblasts were exciting. As shown in Figure 16, the growth of HMEC from a carcinoma sample was significantly inhibited by E_2 when grown on collagen Type 1 but not when grown on mitomycin-C treated fibroblasts. However, E_2 did inhibit the growth of HMEC from a fibroadenoma grown on mitomycin-C treated fibroblasts (Figure 15), showing that the inhibitory effects of high dose E_2 are not blocked by the presence of mitomycin-C treated mammary fibroblasts. Further experiments are required to determine if the growth effects of E_2 observed in cells grown on collagen Type I are different in cells grown on mitomycin-C treated fibroblasts. It was expected that growth would be inhibited by high dose E_2 in cells grown on mammary fibroblasts, as *in vivo* growth of mammary tumor cells in breast cancer patients is frequently inhibited by high dose E_2 [Clarke et al., 1990]. The inhibition of tumor growth by high dose E_2 was the rationale for high dose E_2 as a treatment modality.

Haslam's group also demonstrated that in co-culture experiments of stromal and epithelial cells, E_2 also stimulates DNA synthesis in the fibroblasts [Haslam, 1986]. This effect is also dependent on the presence of live epithelial cells. The bidirectionality of E_2 stimulation has also been observed *in vivo* where E_2 stimulation of DNA synthesis is observed to occur first in the stromal cells followed several hours later by stimulation in the epithelial cells [Shyamala and Ferenczy, 1984]. Bidirectionality of these responses is supportive of a E_2 growth response model incorporating the production of a locally diffusible growth factor(s). McGrath [1983] utilized histoautoradiography to demonstrate that in mixed cultures of MEC and mammary fibroblasts from mice, E_2 stimulated DNA synthesis in MEC only where there is direct contact or very close juxtaposition between the fibroblasts and epithelial cells. However, he noted that the growth-stimulatory effect of E_2 is not common to all colonies of cells in the cultures and, at this time, it is difficult to interpret the data presented by McGrath.

The results of the studies on E_2 modulation of HMEC growth presented in this thesis have led to the proposal of a number of important studies to be carried out in the work leading to completion of my Ph.D. research. As already mentioned briefly, I am interested in further pursuing the experiments on the effects of insulin concentration on HMEC growth. If IGF-1 at low concentrations can replace the high levels of insulin required for maintenance of HMEC in serum-free primary culture, then the growth effects of E_2 will be examined in the new medium. The effect of reducing the hydrocortisone levels on E_2 stimulation of growth in low insulin medium will also be examined. Another goal of my future research is also to modify the serum-free medium composition presently used in order to minimize or eliminate the presence of cholera toxin. I will investigate the ability of cAMP or related analogues to replace cholera toxin, if in fact they are required at all, prior to continuation of further growth studies.

The results of the co-culture studies also suggest a number of follow-up studies. It has already been discussed that E_2 stimulation of HMEC growth *in vivo* is preceded by stimulation of growth in the surrounding stromal cells [Shyamala and Ferenczy, 1984] and that E_2-stimulated growth of HMEC *in vitro* is accompanied by DNA synthesis in the fibroblast feeder layer [Haslam, 1986]. This observation supports a critical role of the stromal cells. I am planning to compare the E_2 regulation of growth of HMEC seeded directly on to feeder layers of mitomycin-C treated mammary fibroblasts or irradiated fibroblasts, HMEC seeded on to nontreated mammary fibroblasts and HMEC co-cultured with actively dividing mammary fibroblasts but physically separated from them by microporous collagen-coated filters. This will determine if actively dividing fibroblasts are needed for E_2 stimulation of HMEC growth and if direct contact with the fibroblasts is required or if locally diffusible paracrine growth factors will result in E_2 growth regulation. I am also planning to compare the growth of HMEC grown on feeder layers of mammary fibroblasts from all three types of tissue utilized in this thesis, as it is possible that defects in the stromal response to growth regulatory signals are involved in the deregulation of growth in the epithelial cells comprising the mammary tumor.

2. The Effects EGF and TGF-α on the Growth of HMEC

EGF has been shown to stimulate MEC growth, both *in vivo* [Gardner et al., 1989, Coleman et al., 1988] and *in vitro* [Stoker et al., 1976, Stampfer et al., 1980]. In mice it appears that EGF plays a role in both the initiation and maintenance of the breast cancer process [Kurachi et al., 1985]. Our results indicate that EGF is indeed a potent and direct mitogen for normal, benign, and malignant HMEC in serum-free primary culture. Although the majority of studies examining the effect of EGF and TGF-α on MEC growth demonstrate a growth-stimulatory effect, two studies have shown the opposite results [Ehman et al., 1984; Yang et al., 1986]. Ehman's group observed an inhibitory effect of EGF on the growth of MEC grown on irradiated fibroblasts in serum-containing media. This result is difficult to interpret, particularly in light of the results presented here, showing a growth-stimulatory effect of EGF on HMEC grown on both collagen Type I and mitomycin-C treated fibroblasts. It has been suggested that the irradiated fibroblasts in Ehman's study were perhaps producing growth-inhibitory factors in response to EGF [Imagawa et al., 1990]. The explanation is purely speculative; however, the serum-free medium used in this thesis is the same as that described by Yang et al. [1982]. Using this medium, Yang *et al.* [1986] failed to see any growth-stimulatory effect of EGF, unless the HMEC are cultured in 3-dimensional collagen gels or hydrocortisone is deleted from the medium. The reason for this discrepancy is not clear, given the high degree of similarity in protocol between the experiments in this thesis and those of Yang's group.

The variation in response to EGF among individual samples (167-3455%) is of considerable interest due to the large degree of variability of receptor levels observed in mammary tumor biopsy samples [Nicholson et al., 1988]. Currently we are using the tetrazolium dye-reduction (MTT) assay to measure growth rather than ^{3}H-TdR incorporation, which was used prior to completion of this thesis. The MTT assay uses far fewer cells per

experimental condition, so it is now possible to measure both EGF-binding to EGFR and EGF growth-stimulating effects on HMEC from the small tissue samples that we receive from the operating room. The ability to compare both of these parameters will allow us to investigate if differences in EGF-binding (receptor levels) can explain the large variability in the degree of growth stimulation by EGF.

The variation in magnitude of growth stimulation by EGF may also be due to differences among the samples in the levels of other receptors that also interact directly or indirectly with EGF in stimulating cell growth. Synergism between EGF and IGF-1 has been shown previously in cultures of bovine MEC [Shamay et al., 1988]. The interaction of high concentrations of insulin used in our serum-free medium with the IGF-1 receptor has already been discussed with respect to its potential role in the inhibition of growth stimulation by physiological concentrations of E_2. With regard to the variation in the magnitude of responses to EGF, it is necessary to consider further the possibility that high concentrations of EGF + insulin may mimic the synergistic effect of EGF + IGF-1 observed in primary cultures of bovine MEC [Shamay et al., 1988]. If this is indeed occurring, then variations in IGF-1 receptor levels among individual samples could cause variable degrees of synergism with EGF, which could be a factor contributing to the large variability in the magnitude of growth stimulation by EGF.

TGF-α also significantly stimulates the growth of HMEC in primary culture (Table VIII). However, samples vary as to whether TGF-α is equal to or more potent than EGF in stimulating HMEC growth. At present there is no explanation for the differences in the growth-promoting activity between EGF and TGF-α, considering they are generally thought to act via a common receptor pathway [Korc et al., 1991]. One possibility is that interaction between exogenously added growth factors and endogenously produced factors may occur, with different interactions between EGF and TGF-α and endogenous growth factors Both EGF and TGF-α, as well as numerous other factors such as platelet-derived growth factor, IGF-1, IGF-2,

and transforming growth factor-β, are all likely candidates for such endogenously produced growth factors [Davidson and Lippman, 1989]. Synergism between EGF and IGF-1 has already been demonstrated [Shamay et al., 1988]; however, it was not investigated if TGF-α also synergized with IGF-1. If EGF and TGF-α differ in the degree to which they synergize with IGF-1 (or high concentrations of insulin), then this could account for the differences in EGF and TGF-α growth responses as well. It is worth noting that TGF-α is also a more potent mediator than EGF in the stimulation of both bone resorption and neovascularization [Ibbotson et al., 1986; Schreiber et al., 1986].

Another possibility is that EGF and TGF-α interact in different ways with EGFR [King, 1988]. Relatively little is known about the mechanism of growth stimulation by EGF or TGF-α. The degree of growth stimulation in HMEC as presented in this thesis is considerably greater than that observed in the epidermoid carcinoma A431 cell line usually used to study the mechanistic effects of EGF. A431 cells are growth inhibited by the concentrations of EGF used in this thesis, and are only stimulated at extremely low concentrations of EGF (pg/ml). The inhibitory effect of physiological levels of EGF on A431 is probably due to the extremely amplified levels of EGFR in these cells. Given the finding that HMEC is so significantly growth stimulated by EGF at physiological levels, and the finding that we can successfully subculture HMEC in the presence but not in the absence of EGF in our serum-free cultures (unpublished results, Emerman et al.), our cell culture system should provide an excellent model system in which to investigate the mechanistic effects of EGF and TGF-α in stimulating cell growth.

The two experiments demonstrating a difference in the dose responses of EGF and TGF-α (Figures 8a and 8b) also indicate another variation in the growth stimulatory effects of these two growth factors. The use of the MTT assay will allow us to characterize differences in binding characteristics for both EGF and TGF-α with respect to their growth stimulatory effects.

The results of these experiments in this thesis are the first report of any such differences between EGF and TGF-α in primary cultures of HMEC.

In planning the experiments comparing hormone and growth factor effects in cells from the three different mammary gland tissue types, it was hypothesized that differences among the tissue types would be observed. The results of this thesis show that in general, cells from breast cancers are not different from cells obtained from normal tissue or fibroadenomas in their growth response to E_2 or EGF (Tables IV & VIII). However, it is possible that such differences may occur between normal and cancerous cells from individual samples. By comparing growth responses of normal cells obtained distal to the tumor site and cells from the tumor proper from individual mastectomy samples, it will be possible to address this question directly. Alternatively, gross differences in responses to individual factors may play a small role in malignancy as compared to multiple defects in growth responses to a number of factors. Future experiments investigating the effects of multiple growth factors and hormones alone and in combination will address this issue.

3. The Effects of E_2 plus EGF on the Growth of HMEC

A synergistic effect on growth of E_2 and EGF on cells from several samples was observed (Table IX). This finding gives support to the idea that E_2 may act as a modulator of cellular responses to growth factors, rather than acting directly as a mitogen. The synergism with E_2 and EGF also supports the hypothesis presented earlier that factors absent in the serum-free medium but present in serum-containing medium are required for E_2 growth stimulation *in vitro*. This hypothesis is further supported by the work of Ethier's group who have shown that E_2 can stimulate the growth of rat MEC in a serum-free medium containing 10 ng/ml EGF [Ethier et al., 1987]. However, Ethier's group did not investigate the growth effects of E_2 in serum-free medium without EGF. Although E_2 alone is unable to elicit a growth-

stimulatory effect, it is capable of enhancing the growth-stimulating effect of EGF in some samples. E_2 has been shown to regulate production of IGF-1 in MCF-7 cells [Huff et al., 1988], and EGF and IGF-1 [Shamay et al., 1988] can interact in a synergistic fashion. Therefore the synergistic effect of E_2 and EGF could result from E_2 stimulation of growth factors and these growth factors could then directly synergize with the added EGF. However, it is unlikely that production of IGF-1 accounts for the synergistic effect observed in this thesis. The high concentrations of insulin in the serum-free medium are presumably already activating the IGF-1 receptor, and further effects through the receptor are therefore unlikely. Using antibodies specific to the different growth factors stimulated by E_2, it may be possible to determine the role of various growth factors in the synergism of E_2 and EGF. This strategy is currently being used in an attempt to determine the role of EGF and TGF-α in E_2-stimulated growth of breast cancer cell lines [Clarke et al., 1990]

An E_2-induced increase in EGFR levels could also explain the synergism between E_2 and EGF observed in our studies. Such increases in EGFR have been demonstrated in ER^+ cell lines [Dickson et al., 1986; Bates et al., 1990]. In uterine tissues it has been shown that E_2 is capable of modulating functional EGF and EGFR levels *in vivo* [Gardner et al., 1989; Huet-Hudson et al., 1990]. As already discussed, it is my intention to measure EGF-binding levels in HMEC in serum-free primary culture of HMEC. Investigating EGF binding levels in the presence and absence of E_2 will determine if the synergism between E_2 and EGF is due to modulation of EGF-binding characteristics by E_2.

4. Conclusions and Future Directions

The results of this thesis show that E_2 may be incapable of directly stimulating HMEC growth in a minimally supplemented serum-free culture medium. However, the results of the experiments examining insulin concentration have shown that factors in the serum-free

medium can inhibit E_2 stimulation of growth, and further experiments are required to establish what effects each of the individual supplements have on the growth effects of E_2. In addition to the effects of medium supplements on modulation of E_2 growth regulation, substrate effects have also been demonstrated in this thesis. More complete investigation of the role of stromal cells in E_2 regulation is required before any conclusions regarding the preliminary results in this thesis can be discussed further.

The results of this thesis also show that both EGF and TGF-α are potent growth-stimulatory factors for HMEC from normal, benign and malignant mammary tissue in serum-free primary culture. The results have shown a large degree of variability in the magnitude of growth stimulation in response to EGF among individual samples. However, the different tissue types did not differ significantly in their average responses to EGF. I have proposed experiments to examine the potential causes of the variability among individual samples, as well as additional experiments addressing the question of differences between normal and malignant cells from the same patient. TGF-α is equal to or greater than EGF in its ability to stimulate HMEC growth. Preliminary studies in this thesis have implicated dose-response differences as a potential explanation for the differences in the magnitude of growth responses to EGF and TGF-α. Using the MTT assay, I have proposed experiments to characterize further the differences in dose responsiveness to EGF and TGF-α.

The results of this thesis showing that E_2 can synergize with EGF in the growth stimulation of HMEC in serum-free primary culture support a role for E_2 in growth regulation via the modulation of HMEC responsiveness to growth factors. Alternatively, EGF may be a competency factor required for E_2 stimulation of growth, both *in vitro* and *in vivo*. I have proposed to study the effects of E_2 on EGF binding in order to determine if E_2 alters HMEC responsiveness to EGF via modulation of EGFR levels.

In summary, the results of this thesis have directed my research interests to two specific and independent directions. I am interested in studying and characterizing further the culture conditions which are required for E_2 stimulation of HMEC growth in the absence of serum. Results of such studies may be able to identify the as of yet unidentified serum-factor responsible for conveying E_2 sensitivity to HMEC both *in vitro* and *in vivo*. The other research interest I have developed through completion of this thesis is regarding the nature of growth factor stimulation of HMEC growth. I would like to study the mechanisms involved in the growth regulatory effects observed in response to both the E_2 and growth factors, alone and in combination. The results presented in this thesis have been submitted and accepted for publication in Experimental Cell Research in an article entitled "Hormone and growth factor effectsl on the growth of human mammary epithelial cells in serum-free primary culture."

APPENDIX 1 : Transport Medium

DME:F12	- (1:1)
Hepes buffer	- 10 mM
Calf serum	- 5 %
Insulin	- 5 μg/ml

DME - Dulbecco's Modified Eagles Medium

APPENDIX 2 : Freezing Medium

DME	- 50 %
Calf serum	- 44 %
Dimethylsulfoxide	- 6 %

APPENDIX 3 : Dissociation Medium

DME:F12	- (1:1)
Hepes buffer	- 10 mM
BSA	- 2 %
Insulin	- 5 μg/ml
Collagenase	- 300 U/ml
Hyaluronidase	- 100 U/ml

BSA - bovine serum albumin

APPENDIX 4 : Attachment Medium

DME:F12	- (1:1)
Hepes buffer	- 10 mM
Pooled normal HuS	- 5 %
Insulin	- 5 μg/ml

HuS - human serum

APPENDIX 5 : Preparation of Rat Tail Collagen

The collagen solution was prepared from rat tails by first placing the rat tails in 95 % ethanol for 15 min. The tendons were dissected out and teased apart using scalpel blades and forceps, weighed and place in a 60 mm Petri dish containing sterile deionized water and exposed to ultraviolet light in the laminar flow hood for 24 h. The fibers were then suspended in a dilute acetic acid solution (0.01 N) and stirred at 4^0C for 48 h. They were left for another 24 h in the dilute acid solution without stirring at 4^0C. The solution was transferred into 50 ml ultracentrifuge tubes and spun in a Sorvall ultracentrifuge for 30 min at 10,000 x g. The supernatant consisted of the collagen solution and was bottled and stored at 4^0C.

APPENDIX 6 : Preparation of Pooled Normal Human Serum

Serum samples were collected in the mornings from patients who had fasted over the previous 8-12 h. Blood, received in non-heparinized tubes, was incubated for 30 min at 37° C, then centrifuged at 100 x g and the serum collected. Pooled serum was stored at -20° C.

APPENDIX 7 : Serum-free Medium

DME:F12	- (1:1)
Hepes buffer	- 10 mM
BSA	- 5 mg/ml
Cholera toxin	- 10 ng/ml
Hydrocortisone	- 0.5 μg/ml
Insulin	- 10.0 μg/ml

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