

Review

Host microenvironment in breast cancer development

Epithelial-cell-stromal-cell interactions and steroid hormone action in normal and cancerous mammary gland

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Abstract

Mammary epithelial cells comprise the functional component of the normal gland and are the major target for carcinogenesis in mammary cancer. However, the stromal compartment of the normal gland and of tumors plays an important role in directing proliferative and functional changes in the epithelium. *In vivo* and *in vitro* studies of the murine mammary gland have provided insights into novel stroma-dependent mechanisms by which estrogen and progesterone action in the epithelium can be modulated by hepatocyte growth factor (HGF) and the extracellular matrix proteins, collagen type I, fibronectin and laminin. *In vitro* and *in vivo* studies of estrogen receptor positive, estrogen-responsive human breast cancer cells have also demonstrated that estrogen responsiveness of tumor cells can also be modulated by extracellular matrix proteins, collagen type I and laminin.

Keywords: estrogen, extracellular matrix, growth factors, mammary stroma, progesterin

Introduction

Mammary gland growth and development are mediated through the complex interactions of steroid hormones, polypeptide hormones, growth stimulatory factors and growth inhibitory factors. Normal development and function of the mammary gland are also dependent upon complex interactions between epithelial cells and stromal cells [1,2]. Stromal cells can regulate the epithelium by the production of soluble growth stimulatory and/or inhibitory factors; and components of the extracellular matrix such as collagens, fibronectin and laminin can also act as signaling molecules for epithelial cells, via specific integrins on epithelial cells. Epithelial cells also secrete factors that influence proliferation and function of adjacent epithelial and stromal cells (Fig. 1).

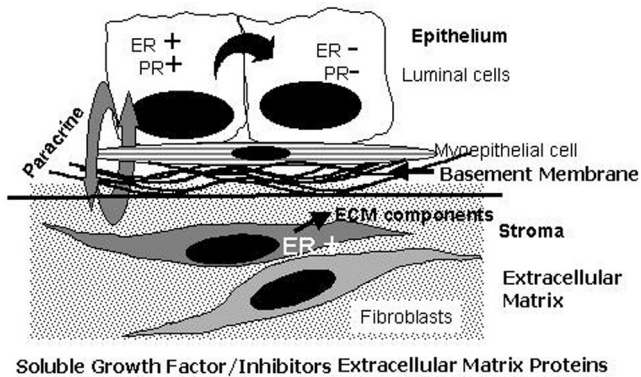
Although there have been numerous studies of signalling mediated by the extracellular matrix and integrin in normal mammary gland and breast cancer cell lines, none has addressed the role of stroma in mediating and modulating steroid hormone action. There is increasing evidence that

a number of responses to estrogen and/or progesterone in the mammary gland may be mediated indirectly through paracrine effects. This review focuses on recent studies from our laboratory addressing interactions between epithelial cells and stromal cells and between steroid hormones and growth factors in the normal murine mammary gland and in human breast cancer cells.

Steroid hormones and mammary gland development

Estrogen and progesterone are required for proliferation and morphogenesis of the normal mammary gland. Estrogen drives ductal development during puberty, whereas estrogen+progesterone mediate the proliferative and morphological changes of ductal side-branching and alveologenesis that occur at sexual maturity and during pregnancy [1,2]. Progesterone is also mitogenic in the premenopausal and postmenopausal human breast [3]. The greater risk of breast cancer in postmenopausal women receiving combined estrogen plus progestin hormone replacement therapy than in those receiving

BrdU = bromodeoxyuridine; Col 1 = collagen type 1; ECM = extracellular matrix; EGF = epidermal growth factor; ER = estrogen receptor; FCM = fibroblast-conditioned medium; FN = fibronectin; HGF = hepatocyte growth factor; IGF-1 = insulin-like growth factor I; LM = laminin; PBS = phosphate-buffered saline; PR = progesterone receptor; R5020 = promegestone.

Figure 1

Model of epithelial-cell-stromal-cell interactions. ECM, extracellular matrix; ER, estrogen receptor; PR, progesterone receptor.

estrogen alone indicates a significant role for progesterone in mammary carcinogenesis [4].

Mammary stroma and estrogen-induced proliferation and morphogenesis in the epithelium

Estrogen receptors (ERs) are expressed in both epithelial and stromal cells [5], and certain estrogenic effects in the epithelium are modulated by mammary stroma both *in vivo* and *in vitro* (review [1]). Studies in both rodent and human mammary tissues have shown that markers of proliferation such as Ki67, proliferating-cell nuclear antigen or BrdU incorporation and ERs are rarely colocalized in the same epithelial cells, suggesting that the proliferating epithelial cells are not the ER⁺ cells [6,7]. Studies of mice from which the ER α gene has been deleted indicate that the presence of ER⁺ stroma is needed for an estrogen-induced proliferative response of the epithelium [8].

To investigate the mechanism of the effects of estrogen-dependent stroma in the mammary gland, we have studied murine mammary epithelium *in vitro* using a minimally supplemented, serum-free, three-dimensional collagen-gel primary cell-culture system. We found that ER⁺ mammary fibroblasts can mediate estrogen-induced proliferation in mammary epithelial cells via stroma-derived hepatocyte growth factor (HGF) [9]. Conditioned medium from mammary fibroblasts (FCM) or coculture with mammary fibroblasts causes increased epithelial-cell proliferation and induces a tubular/ductal morphology (Fig. 2a,b). HGF was identified as the mediator of this effect, since the proliferative and morphogenic activity in FCM is completely abolished by neutralizing antibody to HGF but not by neutralizing antibodies to epidermal growth factor (EGF) or insulin-like growth factor 1 (IGF-1) [10]. Although HGF is constitutively produced by mammary fibroblasts *in vitro* under our culture conditions, its production is increased

by treatment of such cultures with estrogen. In contrast, direct addition of estrogen to epithelial cultures produces neither a proliferative nor a morphological response, despite the presence of ER (Fig. 2c). This suggests that *in vivo* the proliferative effects of estrogen may be mediated indirectly by HGF. It is also possible that *in vivo* regulation of HGF production is more complex than observed *in vitro* and may be controlled by both inhibitory and stimulatory factors. Because ER α and β are both present in mammary fibroblasts under these culture conditions, we do not yet know which ER isoform mediates HGF regulation. EGF or IGF-1 also effectively induces proliferation in cultured epithelial cells (Fig. 2d), but these growth factors each produce a different morphology from that produced by FCM or HGF. Whether a similar mechanism is operative in the human breast is not known; however, HGF is produced in normal breast stroma [11].

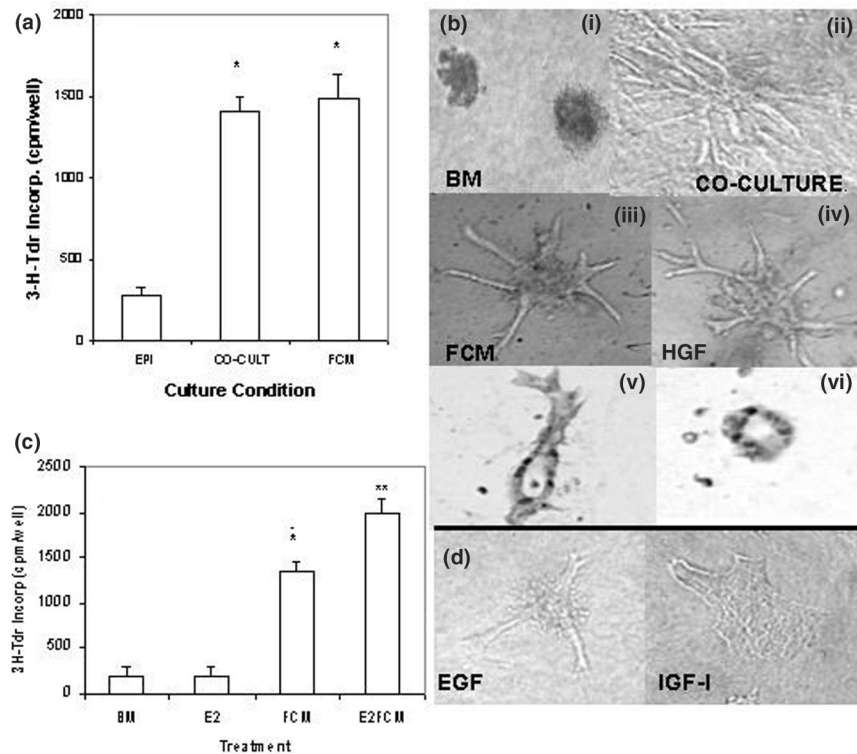
Mammary stroma and progestin-induced proliferation and morphogenesis in the epithelium

That expression of progesterone and of progesterone receptor (PR) in the epithelium is required to induce ductal side-branching and alveologenes in the adult mammary gland and during pregnancy has been confirmed by the absence of alveologenes and lactational function in the mouse from which the PR gene has been deleted [12,13]. Although PRs are present in epithelial cells at puberty, the pubertal mammary gland is much less responsive than the adult mammary gland to the proliferative and alveologenic actions of progesterone [2]. Responsiveness to progestin can be precociously induced in pubertal epithelial cells surgically recombined with adult mammary stroma [14]. Thus, mammary stroma also influences responsiveness of the epithelium to progestin.

Using the culture system described above, we investigated stromal influences on progestin-dependent proliferation and alveologenes in adult murine mammary epithelium [10]. We find that the synthetic progestin R5020 (promegestone) fails to induce proliferation of epithelial cells when added either by itself or with estrogen (Fig. 3a). However, when progestin is added with FCM, it increases the proliferation of epithelial cells to more than that seen in conditioned medium alone (Fig. 3b). The active growth factor in conditioned media with which R5020 interacts is HGF (Fig. 3c,d).

Of particular interest is the unique morphology observed in HGF+R5020-treated cultures. HGF on its own induces extensive formation of duct-like tubular structures (see Fig. 2b), whereas R5020 on its own produces cyst-like structures containing a single lumen (see Fig. 3c). Treatment with HGF+R5020 reduces tubule formation and induces the formation of multiluminal alveolar-like structures, similar to that observed in response to progesterone

Figure 2

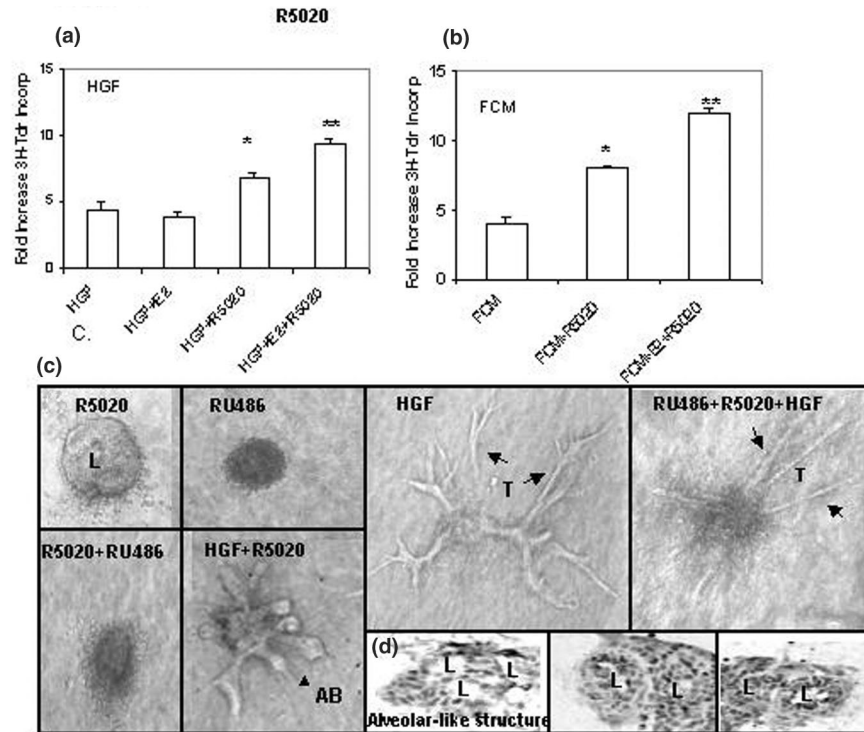


Response of murine mammary epithelial cells to co-culture with mammary fibroblasts and to fibroblast conditioned medium. **(a)** Proliferation of epithelial cells cocultured with mammary fibroblasts or in the presence of conditioned medium obtained from mammary fibroblasts. Murine mammary epithelial cells were suspended in collagen type I gels and cultured alone in basal medium (EPI), over mammary fibroblasts in basal medium (CO-CULT), or in the presence of fibroblast-conditioned medium (FCM). ^3H -thymidine incorporation into DNA was assayed after 3 days of culture. $*P = 0.01$ that proliferation is greater under coculture condition and in the presence of FCM. **(b)** Phase-contrast photomicrographs (i–iv) and histological sections (v,vi) showing the tubular/ductal structure of organoids of epithelial cells in collagen-gel cell culture. Mammary epithelial cells were cultured alone in basal medium, cocultured with mammary fibroblasts in basal medium (CO-CULTURE), cultured alone in the presence of fibroblast-conditioned medium (FCM), or cultured in the presence of 50 ng/ml HGF (HGF) for 3 days. $\times 100$ (i–iv), $\times 400$ (v,vi). **(c)** Effect of FCM and estrogen on proliferation of epithelial cells. Mammary epithelial cells were cultured alone in collagen type I in basal medium, in the presence of 20 nM E_2 , in FCM, or in FCM obtained from fibroblasts cultured in the presence of 20 nM estradiol (E_2FCM); to block any effect of estradiol in the epithelial cells, 200 nM of the antiestrogen, ICI 182,780, was added to epithelial cells at the same time as E_2FCM was added. In the presence of BM (i) only solid spheres were observed. Coculture with fibroblasts (ii), or treatment with FCM (iii), or HGF (iv) produced organoids with a tubulo/ductal morphology. Organoids cultured in the presence of EGF or IGF-1 (d) produced a flattened, sheet-like morphology with few or no tubules. $*P = 0.01$ that proliferation in the presence of FCM was greater than in basal medium or in the presence of E_2 , $**P = 0.05$ that proliferation in the presence of E_2FCM was greater than with all other treatments. **(d)** Morphological response of mammary epithelial cells to EGF (50 ng/ml) or IGF-1 (100 ng/ml). Phase-contrast photomicrographs of epithelial cells were taken on day 3; $\times 100$. BM, basal medium; cpm, counts per minute; E_2 , 17 β -estradiol; EGF, epidermal growth factor; FCM, fibroblast-conditioned medium; HGF, hepatocyte growth factor; IGF-1, insulin-like growth factor I; Tdr, thymidine.

treatment of adult mammary gland *in vivo* (Fig. 3d). The proliferative and morphological responses to R5020 (lumen formation, alveogenesis) are progestin-specific and can be inhibited by the antiprogestin RU486 (Fig. 3d). Analysis of apoptosis in progestin-treated organoids shows that apoptotic cells are localized within the center of the epithelial organoids. This is in contrast to control-treated organoids, in which apoptotic cells are located on the outer periphery of the organoid [10]. This topographically localized apoptotic effect of R5020 within the organoid suggests that this may be the mechanism by which progestins cause lumen formation and that progestins may also play a key role in lumen formation in the mammary gland.

Treatment with progestin and EGF or with progestin and IGF-1 produces no increased proliferation or alveogenic response [10]. Treatment with R5020+EGF does not increase proliferation above EGF alone. The potent mitogenic activity of IGF-1 by itself is halved by the addition of R5020 [10]. These results indicate that progesterone interacts differently with HGF, EGF and IGF-1. Thus, the effects of progesterone at different stages of mammary gland development may be modulated by differential expression of specific growth factors.

In human and mouse mammary glands, HGF is expressed only in mammary fibroblasts, and the HGF receptor, Met, is

Figure 3

Effect of R5020 plus HGF on proliferation of epithelial cells. Murine mammary epithelial cells were suspended in collagen type I gels and cultured in (a) HGF alone (HGF, 50 ng/ml) or with HGF in combination with E₂ (10 nM), R5020 (20 nM) or E₂+R5020 (10 nM+20 nM) or (b) in FCM with or without R5020 or E₂+R5020. ³H-thymidine incorporation into DNA was assayed after 3 days of culture. The data are expressed for suspensions in basal medium as ³H-thymidine incorporated per well and for HGF- and FCM-treated groups as fold increase over basal-medium control. **P* = 0.05 that proliferation is greater in HGF+R5020 group than in HGF or HGF+E₂. ***P* = 0.01 that the fold increase in proliferation in suspensions in HGF+ E₂+R5020 and FCM+ E₂+R5020 is greater than in all other groups within the same experiment. (c) Phase-contrast photomicrographs of epithelial cell organoid morphology in collagen gel cell culture after 3 days in basal medium containing R5020, RU486, HGF, R5020+HGF, RU486+R5020, or RU486+R5020+HGF. ×100. Note appearance of lumens (L) and alveolar buds (AB) in R5020 and R5020+HGF-treated cultures, respectively, and long tubules (T) in HGF and RU486 +R5020 +HGF-treated cultures. No lumen or alveolar bud formation was observed in the presence of RU486. (d) Histological sections of three separate alveolar-like organoids obtained from cultures treated with HGF+R5020; note presence of multiple lumens (L) within these structures. AB, alveolar bud; E₂, 17β-estradiol; FCM = fibroblast-conditioned medium; HGF = hepatocyte growth factor; T, tubule.

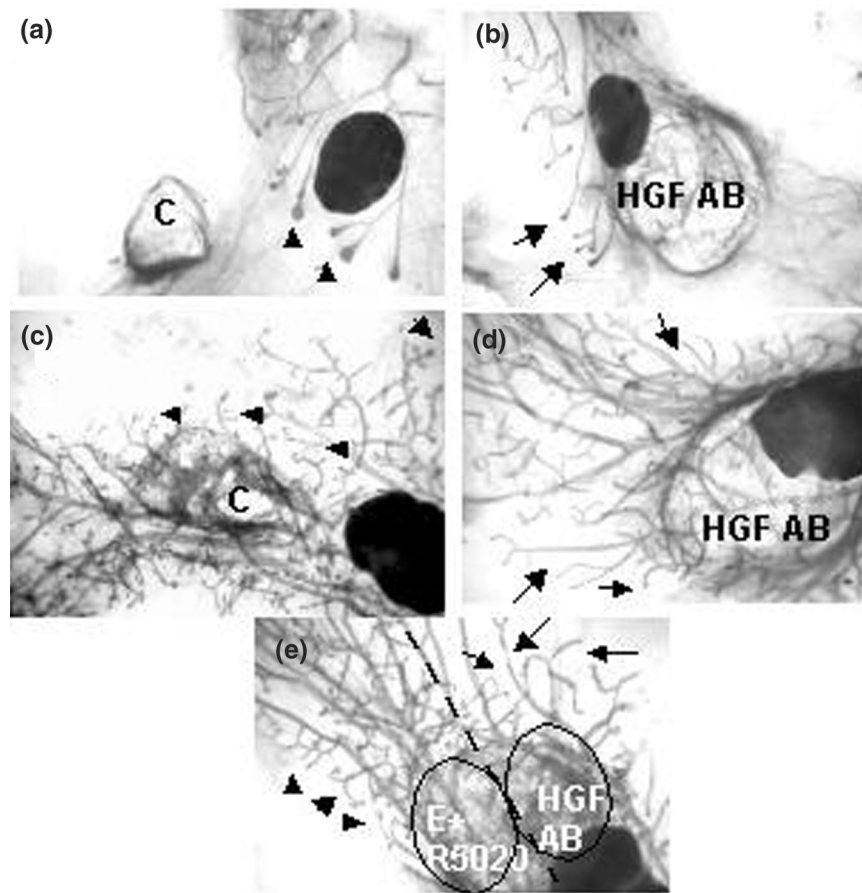
expressed only in epithelial cells [11,15]. In mouse mammary gland, HGF expression starts to rise at midpuberty (6 weeks of age) and is maximally expressed at sexual maturity (12 weeks of age), when the mammary gland is poised for progesterone-induced ductal side-branching and alveolar development. HGF is also present during pregnancy, when maximal alveolar development occurs, and declines to prepubertal levels during lactation. Thus, the *in vivo* pattern of HGF expression is compatible with effects on ductal elongation and alveologenes. To test the effects of endogenous HGF on ductal development and alveologenes *in vivo*, neutralizing antibody to HGF was implanted directly into the mammary glands of pubertal or adult mice. In pubertal mice with intact ovaries, HGF antibody inhibits ductal elongation (Fig. 4a,b). Adult mice with intact ovaries were given two implanted Elvax pellets side by side, one containing estrogen+R5020, the other

containing anti-HGF antibody (Fig. 4e). Alternatively, an Elvax pellet containing anti-HGF antibody was implanted in the right inguinal mammary gland and a control Elvax pellet in the contralateral gland, and the mice were then given daily injections of estrogen+progesterone for 6 days (Fig. 4c,d). In both cases the ductal side-branching induced in response to treatment with estrogen plus progestin was inhibited by anti-HGF antibody. Thus, in the adult gland, HGF appears to play important roles in both ductal elongation during puberty and ductal side-branching, the initial stage in alveolar development, in the adult gland.

Extracellular matrix and integrin-mediated hormone interactions in normal mouse mammary gland

Mammary stroma can also influence the behavior of epithelial cells by altering the composition of extracellular

Figure 4

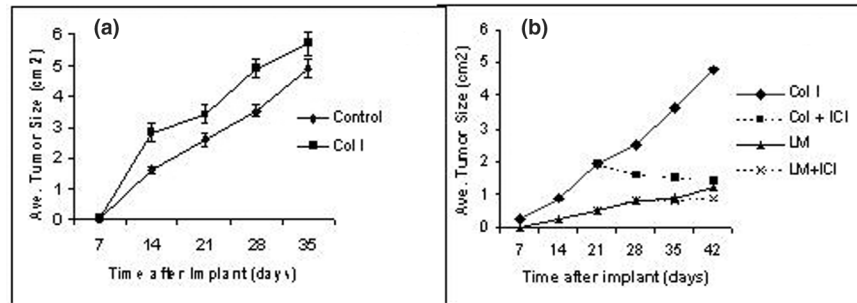


Representative photomicrographs of mammary-gland whole mounts after implantation with neutralizing antibody to HGF. Immature, 5-week-old (a,b) or adult, 12-week-old (c,d) female mice were given implants of Elvax pellets containing anti-HGF antibody (HGF AB) (4 μ g/implant) in the right inguinal mammary gland (b,d) or a control (C) Elvax pellet containing normal serum in the contralateral left inguinal gland (a,c) and were then given daily injections of estrogen + progesterone for 6 days. In addition, adult 12-week-old female mice were given two Elvax pellets implanted side by side, one containing estrogen+R5020, the other containing HGF AB (e). In all cases, whole mounts were prepared 7 days later. Note reduced size of endbuds (indicated by arrows) in the immature gland with implanted HGF AB (b) in comparison with the control (C)-implanted gland in (a) (arrowheads). Note the presence of side-branches in control (C)-implanted adult gland (c) (arrowheads) and their absence in glands with HGF AB implants (d) (arrows). Note the presence of side-branches near the estrogen+R5020 implant (indicated by arrowheads) and their absence near the antibody implant (e) (arrows). C, control; E, estrogen; HGF, hepatocyte growth factor; HGF AB, anti-HGF antibody.

matrix (ECM), which affects signaling pathways mediated by integrins. *In vitro* studies of the effects of ECM proteins in mammary epithelial monolayer cultures derived from adult mammary gland have been reviewed recently [16]. Interactions between steroid hormones and growth factor and between two growth factors are influenced by ECM composition. Collagen type I (Col I) and fibronectin (FN) and, to a lesser extent, laminin (LM) promote an EGF+IGF-1 synergistic effect on proliferation. No synergistic, additive or inhibitory effects of progestin or estrogen with growth factors are observed on Col I or FN. However, on LM, progestin reduces the proliferative response to growth factors [17]. This suggests that signaling pathways specific to ECM integrin can alter interactions between steroid hormones and growth factors.

We have also investigated the hormonal regulation and functional role of ECM proteins and integrins *in vivo* during mammary gland development [18]. Of Col I, LM and FN, only FN levels change appreciably, increasing threefold between puberty and sexual maturity and remaining high during pregnancy and lactation. FN levels are hormonally regulated by estrogen and progesterone. The FN-specific integrin $\alpha_5\beta_1$ is also developmentally and hormonally regulated. It increases twofold between puberty and sexual maturity but decreases during late pregnancy and lactation. The $\alpha_5\beta_1$ integrin level is also decreased by ovariectomy and restored by treatment with estrogen+progesterone. The high levels of $\alpha_5\beta_1$ expression during periods of steroid-induced proliferation indicate that FN signaling may be required for

Figure 5



Growth of MCF-7 cells as tumors in nude mice. MCF-7 cells were mixed with PBS (control) (a) or Col I (a,b) or LM (b) and implanted subcutaneously in ovariectomized nude mice supplemented with estradiol. (b) Mice were subsequently divided into two groups, which received either estradiol (Col I, LM) or estradiol + antiestrogen (ICI 182,780) (Col + ICI, LM + ICI). Col I, collagen type 1; LM, laminin; ICI, ICI 182,780.

hormone-dependent proliferation. Furthermore, the specific temporal pattern of integrin, and growth factor expression relative to the temporal patterns of estrogen-directed vs progesterone-directed morphogenesis, suggest that integrin-mediated signaling may interact with growth factor and steroid hormone signaling pathways to modulate their effects on proliferation and morphogenesis.

Laminin and estrogen-dependent growth in human breast cancer cells *in vitro* and *in vivo*

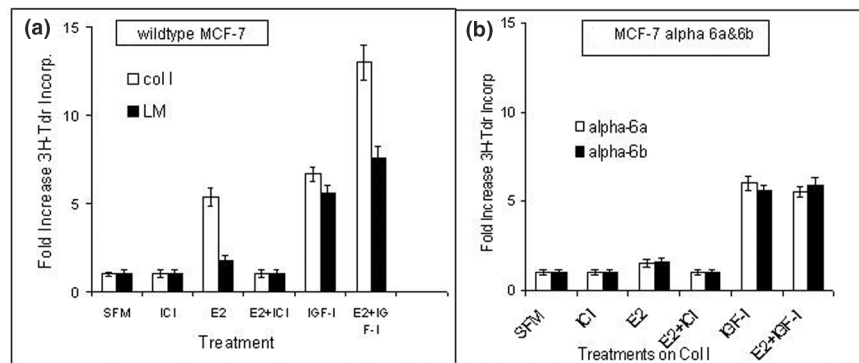
In breast cancer, LMs have been shown to play an important role in progression and metastasis. Several groups have demonstrated that expression of the α_6 subunit of the LM-specific integrin is associated with breast cancer progression, aggressive disease and substantially reduced survival [19–21]. We have shown that LM modulates estrogen action in ER⁺ breast cancer cells *in vitro* [22]. Estrogen induces significant proliferation in both ER⁺ MCF-7 and T47D cancer cells when cultured on Col I or FN, but not on LM. Even though ER levels and ER binding are not altered on LM, estrogen response element activation by estrogen is significantly decreased on LM. Interestingly, inhibition of proliferation by LM is specific to estrogen, since IGF-1 and EGF are able to induce proliferation of MCF-7 cells similarly on LM, Col I and FN, suggesting that proliferation of these cells is mediated primarily by growth factors.

In vivo studies have been carried out to determine the effect of LM on MCF-7-derived solid tumors. MCF-7 cells were implanted subcutaneously with no ECM (PBS control), or with soluble Col I or LM, in ovariectomized nude mice supplemented with estrogen. The LM and Col I groups were subsequently divided in half and either continued to receive estrogen alone or received estrogen+antiestrogen (ICI 182,780). Tumor growth in the presence of estrogen is slightly higher in Col-I-treated tumors than in PBS controls but is significantly lower in

animals with LM-treated tumors (Fig. 5). Importantly, treatment with antiestrogen causes regression of tumors treated with Col I but does not affect those treated with LM (Fig. 5b). Thus, LM inhibits antiestrogen responsiveness as well as estrogen responsiveness in these ER⁺ tumor cells. This is especially relevant because antiestrogen resistance occurs in 30% of ER⁺ primary tumors and is also associated, through undefined mechanisms, with breast cancer progression. Our *in vivo* findings with LM-treated MCF-7 cells suggest that high levels of LM in the tumor ECM might be predictive of ER⁺ primary tumors that will not respond to antiestrogen therapy.

Laminin and Col I can initiate cell signaling via the α_2 integrin subunit. However, the α_6 subunit, which exists as an a or b isoform, confers specificity to LM integrin signaling. Since MCF-7 cells retain estrogen responsiveness on Col I, we reasoned that LM signaling through α_6 might be responsible for loss of estrogen responsiveness on LM. In addition, increased expression of the α_6 integrin subunit has been associated with breast cancer progression. To investigate whether α_6 expression influenced estrogen-responsiveness in breast cancer cells, we stably transfected MCF-7 cells with α_6a and α_6b expressing plasmid constitutively activated by a cytomegalovirus promoter. In contrast to wild-type MCF-7 cells, which proliferate in response to estrogen on Col I (Fig. 6a), we found that neither the α_6a nor α_6b transfectants proliferate in response to estrogen on Col I (Fig. 6b). IGF-1-induced proliferation was significant and was similar to that in wild-type, non-transfected MCF-7 cells; however, there is no additive effect of estrogen+IGF-1 in transfected cells comparable to that seen in wild-type MCF-7 cells. This suggests that LM binding affects the pathway whereby estrogen and IGF-1 signaling converge to promote proliferation. Collectively, these data indicate that LM and the α_6 integrin subunit may be key regulatory components of estrogen-responsiveness and antiestrogen resistance in

Figure 6



Estrogen-responsiveness in MCF-7 wild-type and LM-specific α_6 integrin transfectants. Wild-type MCF-7 cells were plated (50,000 cells per well) on 24-well plates on either Col I or LM (a) and α_6 a and α_6 transfectants were plated on Col I (b) in serum-free medium (SFM). The cells were treated with ICI 182,780 (200 nM) for 24 hours, followed by indicated treatments (17 β -estradiol [E₂] 20 nM, IGF-1 25ng/ml) for 24 hours, labeled with ³H-thymidine for 3 hours and assayed for ³H-thymidine incorporation into DNA. Col I, collagen type 1; E₂, 17 β -estradiol; ICI, ICI 182,780; IGF-1, insulin-like growth factor I; LM, laminin.

certain breast cancer cells, independent of ER expression. The mechanism by which LM induces loss of estrogen responsiveness is under active investigation. We hypothesize that the lack of estrogen-responsiveness of MCF-7 cells adhered to LM is due to LM-specific α_6 receptor/integrin-mediated signaling. The specific downstream mediators of the intracellular signaling pathway activated by LM integrin binding do not provide the appropriate substrate(s) required for estrogen-induced proliferation. Thus, these tumor cells do not respond to estrogen to proliferate, and consequently their growth is not inhibited by endocrine therapy.

Conclusions

In vivo and *in vitro* studies of interactions between epithelial cells and stromal cells in normal murine mammary gland have provided insights into the underlying mechanisms of these interactions to mediate and modulate estrogen- and progesterone-dependent proliferation and morphogenesis. These studies have been extended to potential interactions between human breast cancer cells and surrounding stroma and have led to intriguing findings of ECM-mediated modulation of estrogen-responsiveness in human breast cancer cells *in vitro* and *in vivo*. Further studies are needed to elucidate the mechanisms underlying stroma-mediated influences in breast cancer and may

provide novel therapeutic targets for the treatment of breast cancer.

Competing interests

None declared.

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