EGF controls the in vivo developmental potential of a mammary epithelial cell line possessing progenitor properties

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The bilayered mammary epithelium comprises a luminal layer of secretory cells and a basal layer of myoepithelial cells. Numerous data suggest the existence of self-renewing, pluripotent mammary stem cells; however, their molecular characteristics and differentiation pathways are largely unknown. BC44 mammary epithelial cells in culture, display phenotypic characteristics of basal epithelium, i.e., express basal cytokeratins 5 and 14 and P-cadherin, but no smooth muscle markers. In vivo, after injection into the cleared mammary fat pad, these cells gave rise to bilayered, hollow, alveolus-like structures comprising basal cells expressing cytokeratin 5 and luminal cells positive for cytokeratin 8 and secreting β-casein in a polarized manner into the lumen. The persistent stimulation of EGF receptor signaling pathway in BC44 cells in culture resulted in the loss of the in vivo morphogenetic potential and led to the induction of active MMP2, thereby triggering cell scattering and motility on laminin 5. These data (a) suggest that BC44 cells are capable of asymmetric division for self-renewal and the generation of a differentiated progeny restricted to the luminal lineage; (b) clarify the function of EGF in the control of the BC44 cell phenotypic plasticity; and (c) suggest a role for this phenomenon in the mammary gland development.

Introduction

Phenotypic plasticity and the ability to acquire a motile behavior are essential properties of epithelial cells, known to play an important role in early development, tissue morphogenesis, and tumor progression. Diffusing growth factors, as well as cell–ECM interactions, participate in the control of epithelial cell phenotype. Numerous general and tissue-specific regulatory molecules have been identified, but it remains unclear how their signals are integrated by epithelial cells.

In mammary gland, most morphogenetic and cell differentiation events occur postnatally. In newborn female mouse, the mammary gland consists of a rudimentary system of epithelial ducts embedded in fatty connective tissue. At puberty, the mammary ducts elongate, branch, and progressively invade the entire mammary fat pad. During pregnancy, intensive lateral branching and alveolar development occur, so that at parturition, the mammary fat pad is completely filled with milk-producing alveolar units.

The mammary epithelium is organized into two layers, a luminal epithelium and a basal layer of myoepithelial cells. The bilayer is surrounded by a basement membrane which separates the epithelium from the connective tissue stroma. During lactation, luminal epithelial cells differentiate into secretory cells, whereas basal myoepithelial cells acquire a contractile phenotype. Luminal cells are characterized by expression of the specific cytokeratin pair, keratin (K)*8 and K18, whereas myoepithelial cells express the basal cytokeratins, K5 and K14, along with P-cadherin and smooth muscle contractile proteins (Sonnenberg et al., 1986; Taylor-Papadimitriou and Lane, 1987; Daniel et al., 1995; Deugnier et al., 1995).

Data obtained from the serial transplantation of mammary epithelial fragments strongly suggest that pubescent and adult mammary epithelium, like other epithelial tissues, contains self-renewing, pluripotent stem cells capable of giving rise to lineage-restricted progenitors for luminal and myoepithelial cells (Daniel et al., 1968; Smith and Medina, 1988; Kordon and Smith, 1998; Slack, 2000; Watt and Hogan, 2001).
The phenotype and location of these cells in mammary epithelium remain uncertain. Recent in vitro experiments based on cell separation techniques have suggested that bipotential mammary precursor cells belonged to the luminal compartment (Pechoux et al., 1999; Smalley et al., 1999). Other studies provided evidence that precursor cells in the human mammary gland display basal characteristics (Stingl et al., 2001; Böcker et al., 2002). In addition, the so-called cap cells, located at the edge of terminal end buds (bulbous structures found at the apex of elongating ducts in the pubertal mouse mammary gland) have been proposed to possess important morphogenetic properties and to serve as progenitors for both luminal and myoepithelial cells (Dulbecco et al., 1983; Williams and Daniel, 1983). Finally, extensive ultrastructural studies revealed undifferentiated “pale” or “light” cells resting on the basement membrane or the suprabasal surface of myoepithelial cells. These cells are rare, but they are present at all stages of mammary development and are distributed throughout the mammary tree (Smith and Medina, 1988; Smith and Chepko, 2001).

We recently established and characterized mouse mammary epithelial cell lines (BC20 and BC44) that express high levels of the basal cell markers, K5/K14 and P-cadherin (Deugnier et al., 1999). These basal mammary epithelial cells appear to have a plastic phenotype. Cultured in the absence of EGF, basal cells displayed well-developed cell–ECM contacts and were deficient in cell–cell junctions. Culture in the presence of EGF for 8–10 d resulted in a decrease in focal contact number and the establishment of cell–cell junctions. These morphological changes were accompanied by the down-regulation of basal marker protein and mRNA levels, indicating that, in culture, EGF acts as a negative regulator of the basal cell phenotype. The effects of EGF treatment were reversible because, after the removal of EGF from the culture medium, the cells progressively returned to the basal phenotype, as shown by changes in cell–cell and cell–ECM adhesion and the up-regulation of basal markers (Deugnier et al., 1999). Neither EGF-treated, nor untreated cells expressed detectable amounts of the keratins characteristic of the luminal layer, K8 and K18.
Considering their remarkable phenotypic plasticity, we decided to study the developmental potential of BC44 cells in vivo. We have found that BC44 cells cultured in the absence of EGF after injection into the cleared mouse mammary fat pad, were able to differentiate into luminal, secretory cells and establish a bilayer with luminal and basal cells expressing specific keratins. Long-term treatment with EGF in culture resulted in the loss of the in vivo morphogenetic potential and led to the induction of active MMP2, thereby triggering cell scattering and motility on laminin (LN5). These results reveal that BC44 cells possess luminal lineage-restricted progenitor properties and suggest a role for EGF in the control of the mammary progenitor cell phenotype.

Results

Basal mammary epithelial cells possess an important developmental potential

BC44 cells, either untreated or treated with EGF, were injected into the cleared mammary fat pads of 3-wk-old syngeneic female mice. In both cases, whole-mount staining of the fat pads performed 4 wk after injection revealed solid outgrowths of up to 5 mm in size (unpublished data). Histological analysis revealed that untreated basal cells formed lumen-containing, ramified epithelial structures, whereas EGF-treated cells were predominantly scattered throughout the stroma, with only rare cyst-like structures detected (Fig. 1 A). The epithelial structures derived from untreated basal cells formed a properly polarized bilayer, with a luminal K18-positive and a basal K5-positive cell layer (Fig. 1, B and C). These bilayer structures were surrounded by a basement membrane that contained LN5. In the outgrowths derived from EGF-treated basal cells, epithelial cells, as detected with a pan-keratin antibody, were mostly scattered throughout the stroma, and LN5 deposition was diffuse (Fig. 1 B). These outgrowths contained some epithelial cells that expressed K5 (Fig. 1 B), but no K18-positive cells (unpublished data). We analyzed the ability of untreated BC44 cells to acquire a fully differentiated phenotype, by mating the host mice 6 wk after injection, and dissecting the mammary fat pads containing the outgrowths on the 14th day of pregnancy. The bilayer structures formed by untreated BC44 cells in the pregnant mice contained luminal cells that synthesized β-casein and secreted it into the lumen (Fig. 1 D). α-Smooth muscle actin, a marker of myoepithelial cells, was not expressed in the epithelial bilayer structures established by the transplanted cells in virgin and pregnant animals (unpublished data).

Therefore, we concluded that basal BC44 cells cultured in the absence of EGF retain considerable developmental potential: they can differentiate into luminal, secretory cells, and establish a bilayer with luminal and basal cells expressing specific keratins. In contrast, EGF-treated basal cells displayed low morphogenetic competence, and remained scattered throughout the stroma, displaying an invasive phenotype.

Basal mammary epithelial cells grown in the presence of EGF acquire the ability to migrate on LN5

Cells engaged in morphogenetic processes interact with the ECM. Depending on the developmental context, the ECM may be involved in cell anchorage or may provide a substrate for migration. Therefore, we compared the adhesive characteristics and motility of basal cells grown in the presence and absence of EGF. Cell attachment assays revealed that both untreated and EGF-treated BC44 cells adhered well to LN5, FN, and LN1 and did not attach to CI and CIV. EGF treatment resulted in a small decrease in adhesion to FN. Each bar represents the mean of three independent measurements ± SD. (B) Flow cytometry analysis of integrin levels in untreated (normal lines) and EGF-treated (bold lines) BC44 cells. Untreated and EGF-treated BC44 cells did not express α1 or α2 and displayed similar amounts of α3, αv, α6, β1, and β4 integrin chains. Expression of α5 chain and of the β1 chain epitope recognized by the 9EG7 antibody (β1*) were reduced upon EGF treatment. Dotted lines show negative controls in which the primary antibody was omitted.
Thus, the higher levels of both the small to moderate amounts of reported to be induced upon ligand occupancy or Mn were higher in untreated BC44 cells. The 9EG7 epitope was chain epitope recognized by the 9EG7 monoclonal antibody surprisingly, levels of the large amounts of repertoire. Untreated and EGF-treated BC44 cells produced treatment did not significantly affect the basal cell integrin stimulation of the FN receptor, whereas intercellular interactions were maintained on FN, preventing cells from scattering. Bar, 100 μm.

Flow cytometry analysis showed that long-term EGF treatment did not significantly affect the basal cell integrin repertoire. Untreated and EGF-treated BC44 cells produced large amounts of β1, α3, α6, and β4 integrin chains and small to moderate amounts of α5 and α6, whereas neither the α1 nor the α2 chain was detected (Fig. 2 B). Interestingly, levels of the α5 integrin chain and of a β1 integrin chain epitope recognized by the 9EG7 monoclonal antibody were higher in untreated BC44 cells. The 9EG7 epitope was reported to be induced upon ligand occupancy or Mn2+ stimulation of the FN receptor, α5β1 (Bazzoni et al., 1998). Thus, the higher levels of both the α5 chain and the 9EG7 epitope in untreated BC44 cells may account for the stronger adhesion of these cells to FN (Fig. 2).

We evaluated the ability of basal cells to migrate in response to ECM stimulation by monitoring the dispersion of cellular aggregates deposited on surfaces coated with individual ECM proteins. After 24 h of shaking in culture medium, isolated EGF-treated cells assembled into compact multicellular aggregates, whereas untreated cells formed loose cell clusters (Fig. 3 A). These observations are consistent with our previous work showing that BC44 cells were able to establish firm cell–cell junctions only after treatment with EGF (Deugnier et al., 1999). Cellular aggregates formed by EGF-treated BC44 cells and deposited on ECM protein coated surfaces, after 24 h of contact with ECM, were well spread on FN and LN5, and poorly spread on CIV and LN1. Notably, LN5 induced the dispersion of cellular aggregates, whereas intercellular interactions were maintained on FN, preventing cells from scattering. Bar, 100 μm.

Displayed numerous focal adhesions typical of stationary cells (Fig. 4). In contrast, on LN5, EGF-treated BC44 cells were polarized, with extended lamellipodia and filopodia typical of motile cells, and actin stress fibers and focal adhesions appeared to be much less prominent than on FN. Untreated cells were well spread on FN and LN5, and displayed abundant, well-formed stress fibers and focal adhesions (Fig. 4).

We assessed the migratory behavior of basal cells on ECM proteins directly by monitoring cell migration for 14 h with a time-lapse video recorder. Representative tracks of untreated and EGF-treated BC44 cells plated on LN5 and histograms of cell migration rates are shown in Fig 5. The individual cell tracks clearly indicate marked differences in the migratory behavior of the cells tested. On LN5, the untreated BC44 cells remained stationary or migrated over very short distances (Fig. 5 A), whereas the majority of EGF-treated cells were highly motile, displaying both directional and random movements. Quantitative analysis of the migration assay results indicated that 75% of EGF-treated BC44 cells, and only 14% of untreated cells, migrated on LN5 at speeds >20 μm/h (Fig. 5 B). The rate of 20 μm/h was used as a threshold, above which cells were considered to be essentially motile. Additional experiments demonstrated that BC44 cells, regardless of EGF treatment, were poorly motile on LN1 and stationary on FN (Fig. 5 B). The movement of basal cells on LN5 was inhibited by function-blocking anti–β1-integrin and anti-LN5 antibodies (unpublished data). Thus, basal cells subjected to long-term EGF treatment switched from a stationary to a motile phenotype, and LN5 played a specific role in promoting the migration of these cells.
To study the role of EGFR signaling in LN5-dependent basal cell migration, we first compared the EGFR levels expressed on the surface of basal cells grown in the presence or absence of EGF. Flow cytometry analysis revealed that EGF-treated and untreated cells presented similar levels of EGFR (Fig. 6 A). The aggregate dispersion and migration assays described above (Figs. 3 and 5) were all performed in the absence of EGF. The addition of EGF to the incubation medium in cell migration assays had no major effect on the migratory behavior of BC44 cells on LN5 (Fig. 6 B). These results suggest that ligand-dependent EGFR activation was not involved in the LN5-dependent motility of BC44 cells.

EGF treatment induces the secretion of active MMP2, which is required for the migration of basal mammary epithelial cells on LN5

In a variety of epithelial cell lines, migration on LN5 has been shown to depend on LN5 γ2 chain cleavage by the gelatinase MMP2 (Giannelli et al., 1997; Koshikawa et al., 2000). We used gelatin zymography to compare MMP2 and MMP9 activities in conditioned media obtained from untreated and EGF-treated BC44 cells (Fig. 7 A). Zymograms clearly indicated that unlike untreated cells, EGF-treated basal cells, produced an important gelatinolytic activity corresponding to the active 62-kD and the latent 72-kD forms of MMP2. Treated and untreated basal cells produced moderate amounts of a gelatinolytic activity that could be attributed to the latent 92-kD form of MMP9, and, as seen by
plasminogen zymography, similar amounts of urokinase plasminogen activator (u-PA), revealed as a proteolytic band of 33 kD (Fig. 7 A).

In agreement with the results of gelatin zymography, comparative analysis of the conditioned media from EGF-treated and untreated cells by Western blotting using antibodies specific for pro-MMP2 has revealed that treated cells secreted a higher amount of pro-MMP2 than untreated cells (Fig. 7 B). However, we did not observe any difference in MMP2 mRNA levels in treated and untreated cells using semiquantitative and real-time RT-PCR assays (Fig. 7 C; unpublished data). Similarly, the expression of MT1-MMP, a transmembrane matrix metalloproteinase implicated in the activation of MMP2 and in the cleavage of the LN5 γ2 chain (Strongin et al., 1995; Koshikawa et al., 2000) was not affected by EGF treatment (Fig. 7 C).

The high levels of MMP2 production in EGF-treated basal cells were correlated with migratory behavior, suggesting that LN5-induced basal cell motility was an MMP2-dependent process. Consistent with this, we found that the constitutive migration of EGF-treated BC44 cells on LN5 was inhibited by marimastat, a synthetic inhibitor of MMPs, and significantly reduced in the presence of TIMP2, the natural inhibitor of MMP2 (Fig. 7 D). Moreover, the incubation of LN5 with MMP2 before the migration assay induced motility in untreated BC44 cells (Fig. 7 E).

These data demonstrate that, in basal cells, EGF treatment specifically induced the secretion of active MMP2, and that the extracellular cleavage of LN5 by active MMP2 was required for basal cell migration. The observed up regulation of MMP2 expression occurred at posttranscriptional level.

**EGF treatment concomitantly induces BC44 cell motility, MMP2 production, and loss of the in vivo morphogenetic potential**

To study whether the in vitro phenotypic changes of BC44 cells resulting from EGF treatment, i.e., acquisition of a motile phenotype and MMP2 production, can be correlated with a loss of the in vivo morphogenetic potential, we have analyzed the time course of the EGF-induced phenotypic transition. Cells cultured in the presence of EGF for different time periods were tested for their ability to migrate on LN5, produce MMP2 and form organized bilayered structures in vivo. We have found that the number of motile BC44 cells progressively increased in accordance with the length of EGF treatment (Fig. 8 A). In parallel, BC44 cells gradually increased their production of MMP2, as revealed by gelatin zymography performed with the conditioned media (Fig. 8 B). Overall, although after 2–4 days of culture in the presence of EGF, BC44 cells were able to migrate and produced significant amount of MMP2, their transition was accomplished only by 9 d of culture. Consistently, the morphogenetic potential of BC44 cells progressively decreased with EGF treatment, so that organized structures were observed only in three out of six outgrowths after 2–4 d of treatment, and in three out of nine after 9 d. These results

**Figure 7. Induction of active MMP2 by EGF, and MMP requirement for BC44 cell migration on LN5.** (A) Analysis of the proteolytic activities found in the conditioned media of untreated (−) and EGF-treated (+) BC44 cells. (Top) Gelatin zymogram. EGF-treated cells produce large amounts of 62-kD active and 72-kD latent form of MMP2. The gelatinolytic activities above 83 kD produced by untreated and treated cells correspond to the latent 92 kD form of MMP9 (pro-MMP9). (Bottom) Plasminogen zymogram. Regardless of EGF treatment, BC44 cells produce urokinase, revealed as a proteolytic band at 33 kD (u-PA). Negative images are presented with the proteolytic bands shown in black. (B) Relative expression levels of pro-MMP2 in the conditioned media of untreated (−) and EGF-treated (+) BC44 cells analyzed by Western blotting. EGF-treated cells produce higher levels of pro-MMP2 than untreated cells. (C) RT-PCR analysis of MMP2 and MT1-MMP expression in untreated (−) and EGF-treated (+) BC44 cells. Treated and untreated BC44 cells produce similar amounts of MMP2 and MT1-MMP transcripts. The translation factor, EF1α, was used as control. (D) Inhibitory effects of Marimastat, a synthetic inhibitor of MMPs, and TIMP2, the natural inhibitor of MMP2, on the LN5-dependent migration of EGF-treated BC44 cells. (E) Migration of untreated BC44 cells plated on MMP2-cleaved LN5. Petri dishes coated with LN5 were incubated with active MMP2 or with inactive pro-MMP2 for 3 h before plating the cells. Cleaved LN5 promoted the migration of untreated BC44 cells which were otherwise stationary. (D and E) 25 cell tracks were analyzed in each experiment.
suggest that the loss of the in vivo morphogenetic potential of BC44 cells upon long-term treatment with EGF may be directly related to the acquisition of a MMP-dependent motile behavior.

**Discussion**

Our knowledge concerning the location and molecular characteristics of mammary pluripotent stem cells and the segregation of the two major epithelial cell lineages, basal and luminal, is still very limited. We have isolated a mammary epithelial cell line, BC44, which displays remarkable phenotypic plasticity and morphogenetic properties (Deugnier et al., 1999; this study, Fig. 9). BC44 cells routinely grown in the absence of EGF express exclusively basal markers, K5/K14 and P-cadherin, and no luminal keratins K8/K18. In vivo, when injected into the cleared mammary fat pad of young prepubertal mice, these cells gave outgrowths containing ramified, bilayer structures, consisting of K18-positive luminal cells and basally located K5/K14-positive cells, similar to the small ducts and alveoli of the mammary gland. Furthermore, in pregnant recipient mice, the luminal cells of the ductal and alveolus-like structures in the outgrowths synthesized β-casein and secreted it into the lumen. Notably, the basal cells of the observed bilayer structures expressing K5/K14 did not express the essential smooth muscle marker, α-smooth muscle actin, and were therefore unable to differentiate into myoepithelial cells. Taken together, these results suggest that BC44 cells are capable of asymmetric division, for self-renewal and the generation of a differentiated progeny restricted to the luminal lineage.

BC44 cells are clonal derivatives of the HC11 mouse mammary cell line, which is in turn derived from COMMA-D, isolated from the normal mammary gland of a mouse in midpregnancy (Danielson et al., 1984; Ball et al., 1988). The parental cell line, COMMA-D, in vivo, injected into the mammary fat pad, displayed morphogenetic properties; therefore, it was suggested that this cell line contained a population of mammary epithelial stem cells (Medina et al., 1986). Unlike the highly homogeneous BC44 cell clone expressing exclusively basal keratins, COMMA-D is highly heterogeneous, and consists of cells testing positive for basal and luminal keratins and displaying different morphologies. Further molecular characterization of COMMA-D cells is required to identify the individual cell lineages involved in morphogenesis. To our knowledge, none of the other mammary epithelial cell lines shown to possess morphogenetic properties (i.e., to form branching structures in collagen gel and Matrigel) has been reported to establish a bilayer similar to that found in mammary gland (Berdichevsky et al., 1994; Soriano et al., 1995; Oft et al., 1996; Stahl et al., 1997; Hirai et al., 1998; Niemann et al., 1998).

BC44 cells grown in the presence of EGF lost their morphogenetic potential and ability to differentiate into luminal secretory cells, and remained scattered, after their injection.
into the cleared mammary fat pad. EGF is a ligand of EGFR, a member of the ErbB/type 1 family of receptor tyrosine kinases. In the mouse mammary gland, EGF is expressed in the basal myoepithelial cell layer and in cap cells at a higher rate than in the luminal cells, and is also present in the stroma surrounding growing epithelial ducts (DiAugustine et al., 1997; Coleman et al., 1988). Direct analysis of mammary glands from EGFR-negative mice has not been possible due to embryonic or perinatal lethality (for review see Olayioye et al., 2000). However, several studies carried out in vivo have revealed that signaling via both the stromal and the epithelial EGFR is important for mammary duct development (Xie et al., 1997; Luetke et al., 1999; Wiesen et al., 1999). In particular, the targeted expression of a dominant-negative form of EGFR in the luminal epithelium and the extinction of amphiregulin, an EGFR ligand, result in defective duct growth (Xie et al., 1997; Luetke et al., 1999). Consistently, if delivered in situ, via slow-release plastic implants, EGF was shown to reinitiate duct growth in the resting adult mammary gland, by reestablishing growing buds with a basal cap cell layer (Coleman et al., 1988). Overall, the biological responses of mammary epithelium to EGFR ligands in vivo are not yet well understood.

We studied the effects of EGF treatment on the basal cell phenotype by comparing the adhesive properties and migratory behavior of BC44 cells grown in the presence and absence of EGF. Persistent stimulation of the EGFR signaling pathway (cells cultured in the presence of 10 ng/ml EGF) did not significantly change the integrin repertoire of BC44 cells or the ability of these cells to attach to ECM proteins. However, EGF treatment resulted in acquisition of the ability to secrete large amounts of pro- and active MMP2 and to migrate on LN5. Untreated cells produced only small amounts of this MMP and remained stationary on LN5. Previous experiments performed in vitro and in vivo have demonstrated that MMPs, in particular MMP2, MMP9, and MT1-MMP, are downstream targets of the EGFR signaling pathway (Kondapaka et al., 1997; Miettinen et al., 1999, Kheradmand et al., 2002). MMPs are ECM-degrading proteases responsible for the matrix remodeling required for cell migration during normal mammary development and tumor progression (McCawley and Matrisian, 2001). They have been reported to induce the migration of various epithelial cells on LN5 (Giannelli et al., 1997; Koshikawa et al., 2000). According to the current model, MMP2 and the transmembrane MMP, MT1-MMP, cleave the γ2 chain of LN5, thereby modifying the adhesive functions of LN5 and disturbing molecular interactions with other basement membrane components (Koshikawa et al., 2000; Gagnoux-Palacios et al., 2001). BC44 cell migration on LN5 appeared to be an MMP-dependent process, as it was blocked by Marimastat, a synthetic inhibitor of MMPs, and by TIMP2, a natural MMP inhibitor. The observed induction of pro- and active MMP2 expression upon EGF treatment led us to suggest that migration of BC44 cells on LN5 is mediated by this metalloproteinase; however, as revealed by RT-PCR, BC44 cells express also MT1-MMP. Therefore, it can not be excluded that both MMP2 and MT1-MMP participate in BC44 cell migration on LN5. In addition, MT1-MMP may be involved in the MMP2 activation (Strongin et al., 1995; Kheradmand et al., 2002). Notably, LN5 preparation used in this study consisted of molecules containing the putative MMP2 and MT1-MMP cleavage site (Giannelli et al., 1997; Koshikawa et al., 2000). However, the detailed analysis of the γ2-chain cleavage by EGF-treated basal cells remains to be performed.

Importantly, untreated basal cells, which are normally stationary, acquired the ability to migrate on LN5 if this molecule was cleaved by activated MMP2 before the motility assay. Thus, both autocrine and paracrine processing of LN5 may induce basal cell migration. Finally, LN5, a prominent component of the basement membrane surrounding the mammary epithelial bilayer, appears to possess a unique dual potential, as, on the one hand, it serves for anchorage, and, on the other hand, promotes motility of various types of epithelial cells (Miyazaki et al., 1993; Zhang and Kramer, 1996; Giannelli et al., 1997; Goldfinger et al., 1998; Koshikawa et al., 2000). Accordingly, we found that untreated BC44 were stationary on LN5, whereas after EGF treatment, equipped with active MMP2, they required LN5 for the disruption of cell–cell junctions and for motility. It should be considered that basal cells produce and secrete their own LN5 that may participate in the control of their behavior. EGF has been shown to increase LN5 production in certain human carcinoma cell lines (Mizushima et al., 1996). Our preliminary results suggest that EGF treatment does not significantly alter overall LN5 production by basal cells, but rather results in changes in γ2-chain processing (unpublished data).

It remains unknown whether pluripotent progenitor cells in the mammary gland possess a capacity to migrate. In this context, it is worth mentioning that pluripotent or stem cells of the skin residing in the hair follicle bulge region have been shown to be able to exit from this niche and to migrate along the hair follicle to populate various compartments of the skin (Taylor et al., 2000; Oshima et al., 2001). Several lines of evidence suggest that the basal cell plasticity described in this paper is relevant to the differentiation and morphogenetic events that take place during mammary gland development. MMPs have been reported to play an important role in the branching morphogenesis of primary mammary organoids (Simian et al., 2001). MMP2 has been detected at all stages of mouse mammary development and localized to stromal components and the basal epithelial cell layer (for review see Rudolph-Owen and Matrisian, 1998). Furthermore, the LN5 γ2 chain fragment corresponding in size to that generated by MMP2-cleavage has been shown to be present in the developing mammary gland (Giannelli et al., 1999). Thus, LN5 cleavage by MMP2, and the resulting induction of basal cell migration may occur during mammary gland development, particularly in the growing terminal buds, specific structures that provide a site for a variety of developmental phenomena, including morphogenesis and stromal invasion. The undifferentiated cap cells of the ductal end buds that have been reported to possess a migratory potential (Williams and Daniel, 1983) have a phenotype remarkably similar to that of EGF-treated BC44 cells. Indeed, the cap cells express a very low amount, if any, of basal keratins K5/K14, do not express luminal keratins and are negative for smooth muscle markers (Sapino et al., 1993).
It is conceivable that the embryonic mammary buds already contain the entire progenitor cell pool that serves later to generate the ductal and the alveolar structures in the adult gland. Notably, the basal layer of the mammary buds from 16- to 17-d-old mouse and rat embryos, is comprised by cells possessing a phenotype characteristic of the untreated BC44 progenitor cells, i.e., non-myoeipithelial, but K14-positive (Deugnier et al., 1995; unpublished data). A recent study by Jonkers et al. (2001) has clearly demonstrated that in the mammary gland, K14-positive cells can give rise to both basal and luminal cells, thus proving the existence of the mammary precursor cells expressing basal characteristics. Moreover, a population of putative progenitor cells expressing K5/K14, but negative for K8/K18 and α-smooth muscle actin, was recently described in adult human breast tissue (Böcker et al., 2002).

In conclusion, we report here that BC44 cells recapitulate a large part of the mammary epithelial cell developmental program and, in vivo, after their transplantation into mammmary connective tissue, these cells form branching, lumen-containing, epithelial bilayer structures including secretory cells. Long-term treatment with EGF in vitro leads to a phenotypic transition: induction of active MMP2, acquisition of motility, and loss of morphogenetic potential. Under these conditions, LN5 serves as a scattering agent and a substrate for motility. These results strongly suggest that BC44 cells possess properties of mammary progenitors with a differentiation potential restricted to the luminal lineage, and provide evidence that EGF, and interactions with LN5 are important regulators of their phenotype.

**Materials and methods**

**Cell culture**

Establishment of the basal mouse mammary epithelial cell line BC44 was described in a previous article (Deugnier et al., 1999). Basal cells were routinely grown in RPMI 1640 (GIBCO BRL; Life Technologies) supplemented with 10% FCS (Seromed; Biochrom KG), 2 mM l-glutamine (GIBCO BRL), 5 μg/ml bovine insulin (Sigma-Aldrich), and penicillin-streptomycin (GIBCO BRL). When mentioned, murine EGF (Sigma-Aldrich) was added to the culture medium at a concentration of 10 ng/ml.

**Cell attachment assays**

For attachment assays, 96-well tissue culture plates were coated by incubation overnight at 4°C with 4 μg/ml LNS, or with other ECM proteins, all at a concentration of 20 μg/ml. Nonspecific binding sites were blocked by incubation for 1 h at 37°C with 1 mg/ml heat-inactivated BSA in PBS. 100,000 cells in 100 μl of RPMI were added to each well and allowed to adhere for 30 min at 37°C. After gentle washing, attached cells were fixed with 1% glutaraldehyde for 10 min at room temperature, washed with PBS, stained with 0.1% crystal violet (Sigma-Aldrich) for 20 min, washed with water, and extracted with 0.2% Triton X100 for 10 min. Absorbance was read at 595 nm.

**Cell motility and aggregate dispersion assays**

Freshly trypsinized 10^6 cells were resuspended in culture medium and plated on 35-mm bacterial Petri dishes coated with ECM proteins as described above. The plates were incubated for 2 h at 37°C, unattached cells were gently removed and fresh EGF-free culture medium containing, when specified, function-blocking antibodies, EGF or drugs, was added. Cell migration was monitored at 37°C for 14 h in a humidified atmosphere containing 5% CO2 using time-lapse video equipment. In each experiment, we analyzed the movements of 25–50 cells. As indicated, in some cases the LN5-coated Petri dishes were treated with 100 nM active recombinant human MMP2 (Calbiochem) in Tris buffer supplemented with 0.02% Brij 35, or with 100 nM purified human pro-MMP2 (Chemicon International, Inc.) in PBS for 3–4 h at 37°C.

To prepare cellular aggregates for dispersion assays, 5 × 10^6 trypsinized cells in culture medium were inoculated into a sterile silicon-treated screw-cap Erlenmeyer flask, allowed to equilibrate in a CO2 incubator and transferred to a rotary shaker (75 rpm) for 48 h. Cellular aggregates were collected by centrifugation, rinsed with culture medium, and plated on Petri dishes previously coated with FN, CV, LN1, or LN5. After incubation for 24 h, the cells were photographed with a Nikon phase-contrast microscope.

**Western blotting**

Media conditioned by BC44 cells were prepared as for zymography, and specimens (30 μg of total protein each) were loaded on a 10% SDS-PAGE. After electrophoresis, proteins were transferred to PVDF membranes according to the manufacturer’s instructions (Millipore). Membranes were incubated in 5% milk in PBS-0.1% TWEEN 20 for 1 h at room temperature, in order to prevent nonspecific protein binding, and treated overnight at 4°C with 1 μg/ml of a monoclonal antibody recognizing the latent form of MMP2 (Chemicon International, Inc.). After washing with PBS-Tween membranes were incubated for 1 h at room temperature with horseradish peroxidase-conjugated anti–mouse IgG (Amersham Biosciences) diluted 1:5,000 in blocking solution. Immunoblots were developed using the ECL detection system (Amersham Biosciences).

**RT-PCR assays**

Total RNA was isolated from preconfluent BC44 cell cultures using RNAplus reagent (Q-Biogene). For CDNA synthesis, RNA (1 μg) was reverse transcribed with oligo(dT) primer in a volume of 20 μl using the RT-PCR kit from Takara Biomed. After heat inactivation of reverse transcriptase, volume was adjusted to 100 μl with water, and 2 μl were used for PCR amplification with Dynazyme polymerase (Finzymes). In order to perform semiquantitative PCR, the number of cycles was optimized for each set of primers (35 cycles for MMP2, 26 cycles for MT1-MMP, and 18 cycles for EF1α). The following primers were used: 5′-AGACCATTGCG- GAAGCCCAAG-3′ and 5′-CGTGCCTTCCATCATTAAGG3′ for MMP2 detection; 5′-GTGTCCTATGCTCATCAG-3′ and 5′-TCTGGACATGC- GTCCATACCT-3′ for MT1-MMP detection; 5′-CTGAGCCAAAGTC- TAATATGCC-3′ and 5′-GCCAGGCTTGAGACACAGTGC-3′ for EF1α detection. The RT-PCR products were electrophoresed through a 1.5% agarose gel containing ethidium bromide.

**Immunocytochemical staining and flow cytometry**

Cells were plated at low density on glass coverslips coated with FN or LN5 in 24-well tissue culture plates (TPP) and allowed to spread overnight in EGF-free culture medium. For actin and vinculin staining, cells were fixed with 4% formaldehyde in PBS for 10 min and permeabilised with 0.5% Triton X-100 for 5 min at room temperature. Cells were incubated with primary antibodies for 1 h at 37°C and then with appropriate secondary antibodies for 45 min at 37°C. Cells were fixed for the surface expression of α1, α2, α5, α6, α1, β1, and β4 integrin subunits were harvested using trypsin/EDTA, washed, incubated with primary antibody for 1 h at 4°C, with secondary antibody for 45 min at 4°C, and fixed in 4% formaldehyde overnight before analysis. Cells were analyzed for expression of the α1 integrin subunit or EGF-R were fixed in a mixture of 4% formaldehyde and 0.2% Triton X-100 (ICN Biochemicals) in PBS for 15 min at room temperature before incubation with antibodies. In control samples, isotype control Iggs were added to the incubation medium instead of the primary antibodies. 5,000–10,000 cells per sample were analyzed in a FACScan analyzer (Becton Dickinson) using Cell Quest software.

**Antibodies used for immunofluorescence**

The following primary antibodies were used: CKB1, mouse anti-cytokeratin peptide 14 (Sigma-Aldrich); rabbit polyclonal anti-cytokeratin 5 (BAbCo); KS 18.04, mouse anti-cytokeratin 18 (PROGEN); rabbit polyclonal anti pan-keratin (DAKO); V919, mouse anti-vinculin (Sigma-Aldrich); hamster anti–β1-integrin subunit (Hmβ1-1), rat anti–β1-integrin subunit (9E6G7), hamster anti–α1-integrin subunit (H317/8), hamster anti–α2-inte- grin subunit (HM22), rat anti–α3-integrin subunit (SH10-27), rat anti–α6-integrin subunit (GoH3), hamster anti–α5-integrin subunit (H9.28B), and rat anti–β4-integrin subunit (346-11A; Pharmingen); rabbit polyclonal anti–α3-integrin subunit, a gift from Dr. F. Watt (Imperial Cancer Research Fund, London, UK); mouse anti-EGF (Transduction Laboratories); and rabbit polyclonal anti–laminin 5 (L132; Rousselle and Aumaillé, 1994) and rabbit polyclonal anti–β-casein, a gift from Dr. D. Medina (Baylor College of Medicine, Houston, TX). The following secondary antibodies were used: Alexa 488-conjugated goat anti–rabbit IgG and Alexa 488-conju- gated goat anti–mouse IgG (Molecular Probes); FITC- or Texas red-conju- gated donkey anti–rat IgG, goat anti–hamster IgG, and FITC-conjugated...
goat anti-mouse IgG (Jackson ImmunoResearch Laboratories). Phalloidin-Texas red (Molecular Probes) was used to reveal actin filaments.

**Cell transplantation into cleared mammary fat pads, histology, and immunohistochemistry**

Cleared mammary fat pads were prepared in 3-wk-old female Balb/c mice by surgically removing the developing mammary epithelium of the fourth inguinal glands as previously described (Young, 2000). Confluent BC44 cells grown in presence or absence of EGF were harvested with trypsin-EDTA, rinsed with culture medium, and their concentration was adjusted to 10^5 cells/ml in PBS. Ten μl of EGF-treated or untreated BC44 cell suspensions were injected into the right and left cleared fat pads of the same mouse. 5 or 8 wk after transplantation, the mammary fat pads were removed and prepared for analysis. For whole-mount staining, mammary fat pads were flattened on microscope slides, fixed overnight in Carnoy’s solution (75% ethanol, 25% acetic acid), and stained with carmine alum as described (Faraldo et al., 1998). Whole-mount preparations were photographed, rehydrated overnight in 50% ethanol, dehydrated again, cleared in xylene, and embedded in paraffin. Sections of 5 μm were deparaffinized, stained with haematoxylin and eosin, or processed for immunofluorescence labeling. To obtain frozen sections, freshly dissected mammary fat pads were embedded in Tissue-Tek (Miles Diagnostic Division) and frozen in liquid nitrogen. Before staining, 7-

2.5% Triton X-100 to remove SDS and incubated for 48 h at 37°C. Ten

μl of EGF-treated or untreated BC44 cell suspensions were injected into the right and left cleared fat pads of the same mouse. 5 or 8 wk after transplantation, the mammary fat pads were removed and prepared for analysis. For whole-mount staining, mammary fat pads were flattened on microscope slides, fixed overnight in Carnoy’s solution (75% ethanol, 25% acetic acid), and stained with carmine alum as described (Faraldo et al., 1998). Whole-mount preparations were photographed, rehydrated overnight in 50% ethanol, dehydrated again, cleared in xylene, and embedded in paraffin. Sections of 5 μm were deparaffinized, stained with haematoxylin and eosin, or processed for immunofluorescence labeling. To obtain frozen sections, freshly dissected mammary fat pads were embedded in Tissue-Tek (Miles Diagnostic Division) and frozen in liquid nitrogen. Before staining, 7-

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