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Epithelial Progenitors in the Normal Human Mammary Gland

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The human mammary gland is organized developmentally as a hierarchy of progenitor cells that become progressively restricted in their proliferative abilities and lineage options. Three types of human mammary epithelial cell progenitors are now identified. The first is thought to be a luminal-restricted progenitor; *in vitro* under conditions that support both luminal and myoepithelial cell differentiation, this cell produces clones of differentiating daughter cells that are exclusively positive for markers characteristic of luminal cells produced *in vivo* (i.e., keratins 8/18 and 19, epithelial cell adhesion molecule [EpCAM] and MUC1). The second type is a bipotent progenitor. It is identified by its ability to produce "mixed" colonies in single cell assays. These colonies contain a central core of cells expressing luminal markers surrounded by cells with a morphology and markers (e.g., keratin 14^+) characteristic of myoepithelial cells. Serial passage *in vitro* of an enriched population of bipotent progenitors promotes the expansion of a third type of progenitor that is thought to be myoepithelial-restricted because it only produces cells with myoepithelial features. Luminal-restricted and bipotent progenitors can prospectively be isolated as distinct subpopulations from freshly dissociated suspensions of normal human mammary cells. Both are distinguished from many other cell types in mammary tissue by their expression of EpCAM and CD49f (α6 integrin). They are distinguished from each other by their differential expression of MUC1, which is expressed at much higher levels on the luminal progenitors. To relate the role of these progenitors to the generation of the three-dimensional tubuloalveolar structure of the mammary tree produced *in vivo*, we propose a model in which the commitment to the luminal versus the myoepithelial lineage may play a determining role in the generation of alveoli and ducts.

KEY WORDS: mammary stem cells; colony assays; flow cytometry; cell culture.

INTRODUCTION

The mammary gland is a compound tubuloalveolar gland embedded within an irregular connective tissue. Milk is synthesized within the distally located differentiated acini of the gland and then carried proximally through a series of ramified ducts to the nipple *(1)*. The smallest ducts, the intralobular ducts, together with clusters of alveoli, form the lobules or terminal ductal lobular units (TDLUs).

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Abbreviations used: EpCAM, epithelial cell adhesion molecule; TDLU, terminal ductal lobular unit; TEB, terminal end bud; SMA, smooth muscle actin; SLC, small light cell; K, keratin; MaSC, mammary stem cell; HMEC, human mammary epithelial cell; Ma-CFC, mammary colony-forming cell; Ma-CFC-Lu, luminal-restricted progenitor; Ma-CFC-Me, myoepithelial-restricted progenitor; Ma-CFC-LuMe, bipotent progenitor; CALLA, common acute lymphoblastic leukemia antigen; ESA, epithelial specific antigen; BGA2, histo-blood group antigen H type 2; EGF, epidermal growth factor; FACS, fluorescence-activated cell sorting.

Mammary parenchyma is composed primarily of two types of differentiated epithelial cells. Luminal cells are cuboidal/columnar cells that line both the ducts and alveoli. During lactation, the luminal cells within the distal ducts and alveoli initiate milk production and are called alveolar cells. Myoepithelial cells are specialized contractile epithelial cells that are situated between the luminal cells and the basement membrane. In the ducts, myoepithelial cells form a continuous sheath. In the alveoli, their distribution is sparser and their cytoplasmic processes create a looser, basket-like structure that allows some luminal cells to come in contact with the basement membrane *(2)*.

In females, after the full development of the mammary tree is completed during puberty, cyclical bursts of proliferation (and apoptosis) recur during each menstrual cycle and also following the onset of pregnancy *(3)*. Thus, it may be inferred that the mammary gland maintains functionally intact, exogenously regulated progenitor populations throughout adult female life. Numerous studies have studied the proliferating cells in the mammary epithelium *(4–11)*. These have generally shown that cell divisions are most prevalent amongst the luminal cells, although a low rate of cell division within the basal cell subpopulation is also observed.

Differentiated luminal and myoepithelial cells account for >90% of the epithelial cells present in the mammary gland *(9)*. However, occasional cells with an undifferentiated morphology have also been recognized, and these have been considered as candidates for cells that have progenitor activities. One example is the so-called "cap" cell that lines the terminal end buds (TEBs) of elongating ducts and is exclusive to these structures *(12,13)*. Cap cells have a phenotype that is intermediate between luminal and myoepithelial cells, as indicated by a shared, albeit low level expression of markers associated with both lineages (e.g. vimentin, smooth muscle actin (SMA) and MUC1). Some investigators *(12,13)* have therefore suggested that cap cells are uncommitted mammary epithelial "stem" cells. However, an alternative concept is that these cap cells simply represent myoepithelial progenitors that are responsible for ductal elongation *(14)*.

Another morphologically defined candidate stem cell is the "small light" cell (SLC) *(9)*. Chepko and Smith performed an exhaustive electron microscopic study of the resting mouse and rat mammary epithelium. Based on the mitotic pairs identified, they proposed a hierarchy of 3 division-competent, morphologically distinct cell populations. The most primitive of these was a basally-positioned, putative stem cell present at a frequency of 3% until senescence, by which time these cells disappeared *(15)*. Other morphologically defined cell types considered as candidate mammary stem cells include basal clear cells *(5)* and the keratin 19^- ($K19^-$) epithelial cells found within the luminal layer *in vivo (16)*. The latter were postulated to be stem cells because of their greater frequency in small ducts and lobules, their lack of casein production and the fact that their presence could be associated with sites of more active proliferation during pregnancy. However, all of these studies are compromised by their intrinsically limited correlative nature and inability to definitively associate a particular cell phenotype with its progeny output potential.

Transplantation of mammary cells into cleared mammary fat pads has been used as a functional approach to identify mammary epithelial cells with stem cell properties *(5,17)*. These experiments laid the foundation of the concept of mammary epithelial stem cells (MaSCs), although the single-cell origin of the structures obtained still remained to be established. This verification was subsequently provided by studies of transplants of cells at limiting dilutions, which allowed the clonal nature of the regenerated alveolar, ductal and more complex structures to be confirmed *(18,19)*. These findings further introduced the concept of alveolar-restricted and ductal-restricted as well as pluripotent MaSCs in the adult mouse mammary gland. This assay has also been used to show that MaSCs are present throughout the murine mammary tree, with the highest frequency of MaSCs in the end buds and the lowest frequency of MaSCs in lactating alveoli *(5)*. More recently, the cleared mammary fat pad assay has been successfully used to characterize the phenotype of MaSCs in single cell suspensions of mouse mammary epithelial cells *(20)* and to obtain highly enriched populations of these cells *(21)*. These studies have confirmed the presence in the adult mouse mammary gland of pluripotent, self-renewing MaSC populations that are largely separable from other progenitors detectable by their ability to make colonies of differentiating cells *in vitro* (Stingl *et al.*, manuscript in preparation) and strongly imply the existence of a similar hierarchy in human mammary tissue.

However, the development of an analogous methodology for identifying and characterizing stem cells within human mammary epithelium has been more difficult due to the challenges of developing

a suitable *in vivo* xenotransplant assay. Therefore, in the human arena, greater effort has been placed on attempts to optimize conditions for supporting the growth and differentiation of primitive human mammary epithelial cells (HMECs) seeded at clonal densities *in vitro (22–31)*. This strategy has led to the development of methods for identifying multiple subpopulations of HMEC progenitors, including a multi-potent HMEC with self-renewal ability *(30)*. Here we will focus on the HMECs that form adherent colonies of exclusively luminal or myoepithelial cells or mixtures of these differentiated cellular phenotypes *in vitro*.

DEFINITIONS AND NOMENCLATURE

The defining features of a stem cell are its ability to generate differentiated progeny for the lifetime of the organism. This definition, in turn, usually implies an ability to generate progeny with the same, often multipotent, developmental potential. Since single mammary cells with an ability to renew their luminal and myoepithelial differentiation activity have now been identified in both mice and humans using several methodological approaches, it seems useful to limit the term MaSCs to cells that are shown to have such properties. MaSCs are then distinguished functionally from HMEC progenitors, which are simply defined by their ability to generate more than some minimal number of progeny of one or more lineages. Here we describe assays for HMEC progenitors that generate colonies containing at least four cells (i.e. undergo a minimum of two cell divisions) within 7 days *in vitro* and produce progeny that show exclusive evidence of luminal differentiation, myoepithelial differentiation, or both (see markers described below). Accordingly, as a group, the progenitors of these colonies are referred to as mammary colony-forming cells (Ma-CFCs) and the respective subsets as Ma-CFC-Lu, Ma-CFC-Me and Ma-CFC-LuMe. This adoption of operationally defined nomenclature minimizes the type of confusion that arises, for example, from referring to luminal cells as epithelial cells.

CHARACTERIZATION OF MAMMARY CELL LINEAGES FROM *IN VIVO* STUDIES

Human mammary epithelium has now been analyzed for patterns of expression of a large number of proteins (Table I). SMA *(32)*, keratin 14 (K14) *(38)*, CD10 (common acute lymphoblastic leukemia antigen; CALLA) *(33)*, CD49f (α6 integrin) *(34)*, p63 *(35)*, CD44v6 *(37)* and the intermediate filament vimentin *(36)* are generally localized to cells found in the basal cell layer of the normal human adult mammary epithelium. Exceptions to this list include expression of K14 in luminal cells in the large ducts *(44)* and expression of vimentin in fibroblasts found in the stroma that surround the actual mammary parenchyma *(46)*. Markers useful for identifying luminal cells based on their consistent staining pattern *in vivo* include keratin 18 (K18) *(38)*, K19 *(38)*, CD24 *(39)*, CD133 *(40;* unpublished observations) and MUC1 *(41)*. However, lack of expression of K18/K19 by some luminal cells has been noted *(16,45)*. These aberrant cells are found as isolated cells or small clusters of cells within small ducts and TDLUs.

MUC1 is a highly glycosylated apical plasma membrane protein characterized by variable numbers of tandem repeats *(41)*. Considerable heterogeneity in the MUC1 epitopes expressed on different luminal cells *in vivo* has been revealed by comparing immunostaining results with different anti-MUC1 antibodies *(26,47)*. Hence particular care should be used in identifying the antibody preparation used before interpreting MUC1 expression data. Epithelial cell adhesion molecule (EpCAM; also known as epithelial specific antigen or ESA), is a homophilic

Table I. *In Vivo* Distribution of Markers Commonly Used to Characterize HMECs

Marker	Distribution *in vivo*	Reference
SMA	Myoepithelial	*(32)*
CD10/CALLA	Basal	*(33)*
CD49f (α6 integrin)	Basal + endothelial	*(34)*
p63	Basal	*(35)*
Vimentin	Basal and stromal	*(36)*
CD44v6	Basal	*(37)*
K14	Basal[a]	*(38)*
K18	Luminal[b]	*(38)*
K19	Some luminal	*(38)*
CD24	Apical surfaces of luminal cells	*(39)*
CD133	Apical surfaces of luminal cells	*(40)*
MUC1	Apical surfaces of luminal cells	*(41)*
EpCAM	All cells except myoepithelial	*(25, 42)*
ErbB2	All cells except myoepithelial	*(43)*

[a] Also expressed by some luminal positioned cells in the large ducts (*44*).

[b] Many luminal-positioned cells in large ducts do not express K18 (*45*).

Ca^{2+}-independent cell adhesion molecule specific for most epithelial cells *(42,48)*. EpCAM in normal adult mammary tissue is localized primarily to the basal and lateral cell membranes of luminal epithelial cells *(25,42)*. Stromal cells and myoepithelial cells do not express this protein *(48–49)*. ErbB2 expression shows a similar distribution to EpCAM, with strongest expression on the luminal cells, weak-to-no expression by the myoepithelial cells and no expression by the stromal cells *(43)*. Care should be taken in the interpretation of erbB2 immunostaining patterns since erbB2 can exist in two different forms (apical and basolateral) with different antibodies recognizing the different erbB2 forms *(50)*.

CHARACTERIZATION OF HMEC-DERIVED COLONIES

Normal human mammary tissue (obtained from discarded reduction mammoplasty specimens) can be enzymatically dissociated and filtered to obtain a suspension of viable single cells *(51)*. When these are cultured at clonal densities (<500 cells/cm^2) in a serum-free but growth factor-supplemented medium on an irradiated mouse fibroblast feeder layer (NIH 3T3 cells), ~1% of the HMECs will form a colony of >4 cells within a week. The cloning efficiency increases ~fivefold after the cells have been "precultured" a short period (2–6 days) prior to performing the CFC assays. The increased frequency is likely due to the selective loss during the first several days in culture of nonepithelial cells and terminally differentiated HMECs present in the original suspension. Interestingly, the average frequency of Ma-CFCs has not been found to be associated with donor age *(28,31,52)*. HMEC progenitors will typically divide up to a maximum of 20 times before they senesce *(28,53)*. As senescence becomes imminent, the cells become large, flattened and vacuolated. In cultures of HMECs maintained in serum-free conditions, the proportion of cells exhibiting a myoepithelial phenotype typically increases, with a concomitant loss of cells having a luminal phenotype.

When cells are plated under conditions that support the proliferation and differentiation of Ma-CFCs, a spectrum of different colony types is produced after 6–10 days of incubation. Figure 1 shows examples of the three categories to which these are assigned depending on the prevalence of luminal and myoepithelial cell types present: pure luminal cell (Fig. 1(A) and (B)), pure myoepithelial cell (Fig. 1(C)) and mixed phenotype containing both luminal and myoepithelial cells (Fig. 1(D)–(F)). Individual colonies within each subtype may show considerable variation in size. The majority (~80%) of the colonies obtained in colony assays initiated with minimally cultured HMEC have a mixed phenotype, whereas most of the remaining colonies are pure luminal cell colonies *(28)*.

Pure luminal cell colonies are characterized by a tight arrangement of the cells they contain and have indistinct cell borders and a smooth outer colony boundary. In the smaller colonies, the cells form tightly arranged clumps that give the appearance of rounded spherical structures (Fig. 1(A)), whereas in the larger ones (Fig. 1(B)), the cells at the periphery appear more tightly arranged than those located at the center of the colony. Most of the cells within these colonies express MUC1, K8/18, EpCAM and K19, and do not express K14, CD44v6 and histo blood group antigen H type 2 (BGA2; *28,54,55)*. It is interesting to note that K19 is consistently expressed in these colonies *(28,49,54)*, even though K19 is not a reliable marker of luminal epithelial cells *in vivo (16)*. Cells having a luminal phenotype can be induced to further differentiate into casein-producing cells by the addition of fresh culture medium supplemented with of 1 μg/mL ovine prolactin and 50% Matrigel *(30)*.

Pure myoepithelial cell colonies contain a dispersed arrangement of cells with a characteristic elongated shape (Fig. 1(C)). The dispersal of cells in these colonies reflects the ability of myoepithelial cells to migrate in response to stimulation by epidermal growth factor (EGF) present in the culture medium *(28)*. The cells in these colonies express K14, BGA2, CD44v6, CD49f and CD10, but not MUC1, EpCAM and K19 *(23,28,30,55)*. However, they often fail to express SMA, a component inversely associated with the growth status of myoepithelial cells *(56)*.

Mixed lineage colonies typically contain a central core of cells similar to those found in pure luminal colonies (close cell arrangement expressing MUC1, EpCAM and K19, and lack of expression of K14 and BGA2) and are surrounded by a halo of highly refractile, migratory elongated cells (Fig. 1 (D–F)) similar to those found in pure myoepithelial colonies (expressing K14, BGA2, and CD44v6, but not MUC1, EpCAM and K19) *(25,28,55)*. Interestingly, many of the $K14^+$ elongated cells adjacent to the central core also express K18, a marker traditionally considered to be specific for luminal cells *in vivo*

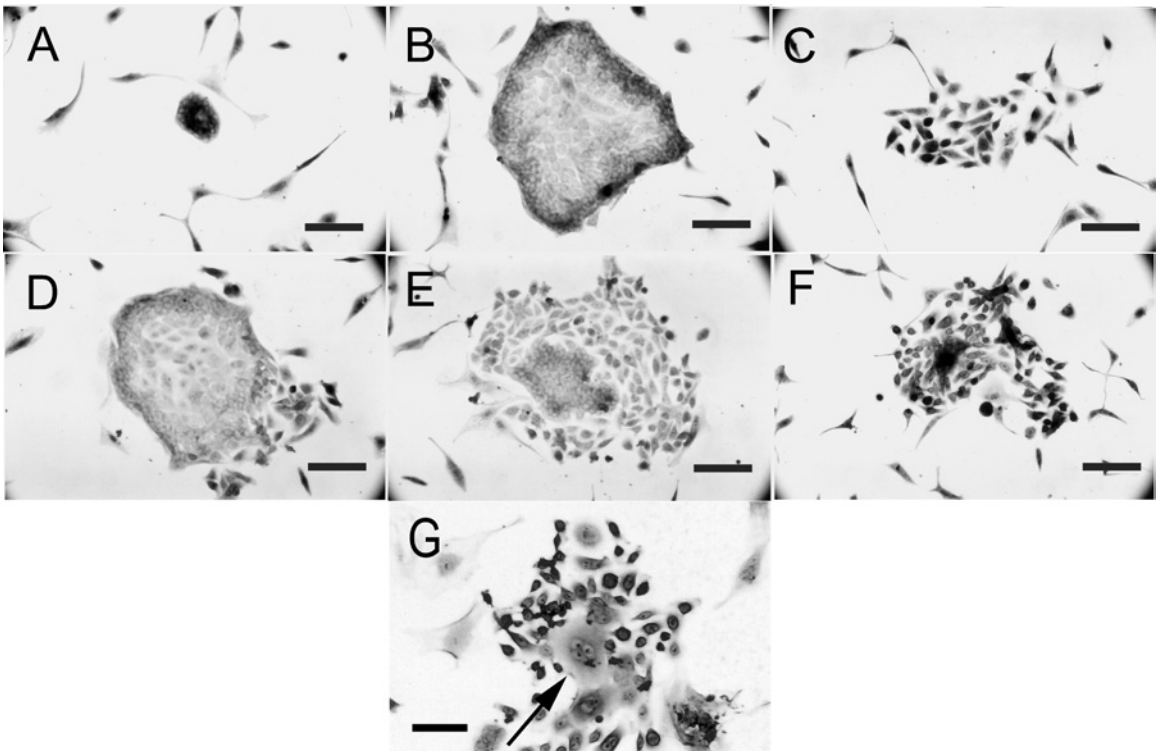

Fig. 1. Range of HMEC colony morphologies observed after 7–10 days in serum-free culture. All colonies in Panels A–F were generated from the same original primary HMEC culture. Colony phenotypes include pure luminal (A–B), pure myoepithelial (C) and mixed (D–F). In some of the mixed and pure myoepithelial cell colonies, the centrally located cells have a squamous morphology and are often multinucleated (arrow in G). Bar = 250 μm.

(38). Similar $K14^+/K18^+$ cells have been described by others *(31,54)* and may represent a morphological intermediate in the generation of myoepithelial cells from a more primitive cell type. Mixed colonies do not represent an artifact of co-plated doublets with different lineage potentialities, as proven by experiments with single cell cultures *(28,31)*.

The centrally located cells in the mixed and pure myoepithelial cell-containing colonies occasionally exhibit a squamous phenotype (Fig. 1(G), arrow), a phenomenon associated with the presence of cholera toxin in the culture medium, cell proliferation and the menstrual cycle status of the individual from whom the sample was obtained *(13,22, 57)*. Cells derived from women who are in the late stages of the menstrual cycle are more susceptible to squamous metaplasia than those in the early stages of the cycle. The centrally located cells in such colonies, particularly when they have a squamous phenotype, are sometimes multinucleated (Fig. 1(G), arrow). The generation of multinucleated mammary epithelial cells has been associated with bypassing "senescence" or "selection," having a methylated $p16^{INK4A}$ promoter, eroding telomeric sequences and a susceptibility to acquire further genomic abnormalities *(53,58)*.

CHARACTERIZATION OF HMEC PROGENITORS

Flow cytometry has been used extensively to characterize the phenotype of CFC-Lu's and CFC-LuMe's progenitors, particularly from HMECs obtained from 2–6-day cultures of dissociated human mammary organoids. As summarized in Table II, this research has shown that most CFC-Lu's have a $MUC1^+/CD133^+/EpCAM^+/CD49f^+/CD10^-/Thy1^-$ phenotype and most CFC-LuMe's have a $MUC1^-/CD133^-/EpCAM^+/CD49f^+/CD10^+/Thy1^+$ phenotype *(25, 28;* unpublished observations). In some freshly isolated cell suspensions, the CFCs are found to be distributed between the $EpCAM^+$ and $EpCAM^-$ fractions (unpublished observations);

Table II. Phenotypic Profiles of Human Mammary Epithelial Progenitor and Nonprogenitor Populations

Marker	CFC-Lu	CFC-LuMe/ Me	Nonclonogenic luminal	Cultured myoepithelial
MUC1	+	− to ±	+	−
CD133	+	−		
EpCAM	+	−[a]	+	−
CD49f	+	+	−	
CD10	−	+	−	
Thy1	−	+	−	
BGA2				+
CD44v6				+
ErbB2	+	+	+	

[a] When using precultured material.

however, after a few days in culture all of the CFCs detected are $EpCAM^+$. By combining immunomagnetic enrichment and multi-parameter fluorescence-activated cell sorting (FACS), CFC-Lu's and CFC-LuMe's can be isolated at purities of ~30 and ~50% respectively (unpublished observations). The $MUC1^-/CD49f^+/CD10^+$ phenotype of CFC-LuMe's suggests that these cells are basally positioned in vivo. This prediction is further supported by immunocytochemical studies of human mammary tissue sections, which have revealed the presence of $MUC1^-/EpCAM^+$ cells in the basal layer of the epithelium *(29)*.

Isolation of the $EpCAM^+/CD49f^-$ fraction yields a population of HMECs that are depleted of CFCs, although in culture loss of either CD49f or EpCAM was found to correlate with decreased CFC activity *(28)*. This scenario is likely associated with the terminal differentiation of HMECs along the luminal and myoepithelial pathways, respectively.

COMPARISON OF MA-CFCs FROM HUMANS AND RODENTS

Several reports have described the presence of CFCs in both mouse and rat mammary cell populations *(59–61)*. Direct comparisons between colonies and CFCs of human and rodent origin is confounded by the different culture conditions and antibodies used for their characterization. Nevertheless, some generalizations can be made. For example, Ma-CFCs from rodent sources appear to make the same three categories of colonies as their human counterparts; i.e., pure luminal colonies and pure myoepithelial colonies as well as mixed colonies containing differentiated cells of both lineages. However, murine progenitors of mixed colonies expressed phenotypic markers shared by luminal cells (mouse milk fat globule membrane antigen), which contrasts with the finding for human CFC-LuMe's that were not found to express markers present at the apical plasma membrane (MUC1 and CD133). Clearly more extensive profiling of these different progenitors from human and rodent sources will be needed to determine the extent of similarity between currently defined compartments.

INTERPRETING COLONY ASSAY DATA

There are several points to be kept in mind when interpreting results of *in vitro* colony assays. First and foremost, although MaSCs may be detectable as CFCs, it is unlikely that all CFCs are MaSCs. In particular, CFCs that display restricted differentiation potentialities and have associated distinct phenotypes are most likely to represent derivative progenitor populations. Not even all bipotent CFCs are likely to be MaSCs if, as in other systems, lineage restriction is a progressive process that can span multiple cell generations.

Another point to be emphasized is that the number and types of cells produced are highly dependent on the culture conditions used. For example, assays of human Ma-CFCs that contain human mammary fibroblasts rather than irradiated NIH 3T3 cells increases the frequency of pure myoepithelial cell colonies and concomitantly reduces the yield of mixed cell colonies *(26,31)*. Altering components of the medium can similarly alter the balance of mature cell types generated, resulting in an apparent shift in the types of CFCs thought to be present originally (unpublished observations).

At present it is not known whether culture conditions that have been optimized to detect different types of Ma-CFCs also support the expansion or even maintenance of human MaSCs. In fact there are several lines of evidence to indicate that this is not the case. First, HMECs maintained *in vitro* only undergo 10–20 cell divisions before they senesce *(28,53)*. This number of divisions is well below the theoretical number of divisions thought to be permissible before telomeric degradation would be limiting and also well below the cell amplification obtained when murine MaSCs are sequentially propagated in cleared fat pads *(19)*. Secondly, HMECs cultured in serum-free media eventually select for cells with a

myoepithelial phenotype *(49,62,63)*. This rapid depletion of luminal cells within the population is not an intrinsic property of the cells since maintenance of HMECs in the presence of HGF, stromal feeders or serum will permit the maintenance of $K18^+/K19^+$ cells *(23,27,49,62,64)*. It is thus possible that serum is required for the sustained maintenance of more primitive types of HMECs, as appears to be the case for human epidermal cells *(65)*. Thirdly, stem cells from many tissues are known to be difficult to maintain *in vitro*, and particularly to expand, in the absence of genetic intervention *(66,67)*. Factors implicated in the maintenance of adult tissue stem cells *in vitro* include guanine nucleotide pools *(68)*, culture substratum *(65,69,70)*, anchorage-independent conditions *(30,71–73)*, presence of serum *(65)* and factors that activate the Wnt signaling pathway *(74)*. In addition, Ehmann and colleagues have demonstrated that rat $K14^+/K18^-$ mammary LA7cells can maintain primary mouse mammary $K8^+K18^+$ cells for at least 30 population doublings *(75)*, thus raising the possibility that epithelial feeders may be essential for stimulating MaSC self-renewal divisions *in vitro*. Interactions between luminal and myoepithelial cells have been demonstrated to be important regulators of epithelial cell polarity *(76)* and epithelial branching morphogenesis *(77)* via laminin-1 and epimorphin production by myoepithelial cells, respectively. It is possible that similar interactions between different subtypes of epithelial cells play a role in maintaining the MaSC niche.

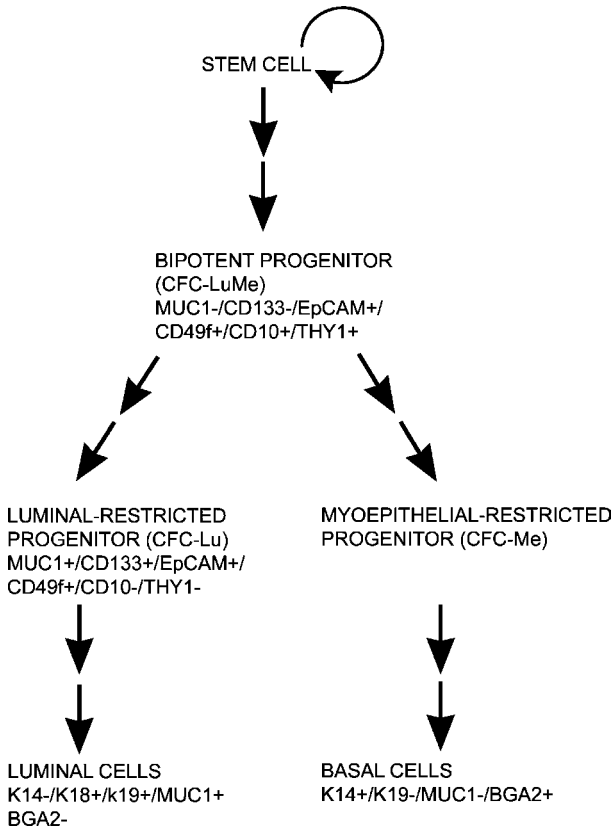

Fig. 2. Proposed hierarchical arrangement of mammary epithelial progenitors.

HIERARCHICAL ORGANIZATION OF HMEC'S AND THE DEVELOPMENT OF ORGANIZED MAMMARY TISSUE *IN VIVO*

Figure 2 presents a schematic representation of the hierarchical organization proposed for HMEC differentiation from a putative MaSC. Assuming that the majority of the progenitors detected in Ma-CFC assays are not MaSCs, CFC-LuMe's would be positioned as their downstream progeny and CFC-Lu's and CFC-Me's as derivatives fully committed to a single lineage. Consistent with this analysis are the findings that CFC-Lu's generate limited numbers, but exclusively CFC-Lu's when replated; the same is true for CFC-Me's *(22,29;* unpublished observations). On the other hand, this argument is weakened by the fact that CFC-LuMe's fail to generate detectable numbers of either CFC-LuMe's or CFC-Lu's upon replating, suggesting, as discussed above, that the earliest steps of HMEC development *in vitro* are not as robust as would be desirable.

The hierarchical schema proposed in Fig. 2 does not, of course, address the question of how these cells generate the three-dimensional mammary tree *in vivo* that consists of organized ducts and lobules or how they may relate to the transplantable ductal- and lobular-restricted mammary progenitors described in the mouse using the cleared mammary fat assay *(18,19)*. Early studies examining the serial transplantation of mouse mammary tissue demonstrated that ductal development decreased with mitotic age of the transplanted cells and not the chronological age of the host *(17,78)*. No *in vivo* data are yet available to make similar assessments of primitive HMECs, although results obtained from plating purified human CFC-Lu's and CFC-LuMe's in three-dimensional matrices may provide some clues. These experiments have demonstrated the formation of colonies that have gross morphologies that resemble alveoli and ducts, respectively *(28,29,79)*.

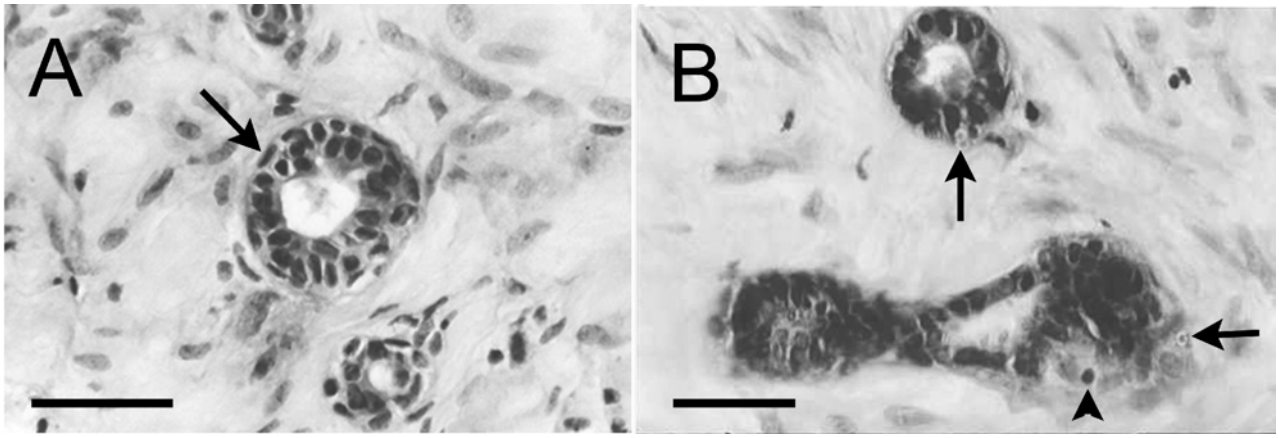

Fig. 3. Epithelial outgrowths observed after 4 weeks *in vivo* when single-cell suspensions of HMEC are engrafted within human mammary fibroblast-containing collagen gels under the renal capsule of female NOD/SCID mice supplemented with exogenous estrogen and progesterone. Note the presence of a stratified epithelium composed of a luminal cell layer and a myoepithelium (arrow in A). Also present are basal clear cells (arrowhead in B) and cells with a SLC morphology (arrows in B). Bar = 50 μm.

Thus a simple model to explain the generation of the mammary tree is that CFC-Lu's represent alveolar progenitors and CFC-LuMe's represent ductal progenitors. In the mixed colonies, cells expressing luminal cell characteristics surrounded by cells expressing myoepithelial cell characteristics are consistently observed *(25,27,28,30,31)*, an arrangement that mirrors that observed *in vivo*.

An alternative possibility is that the type of outgrowth generated in vivo is simply a reflection of the ratio of cells acquiring luminal and myoepithelial fates, and that these may be prefixed or externally controlled in MaSCs. For example, in the presence of factors that promote ductal elongation (i.e., estrogen; *80)*, there may be an increased production of myoepithelial cells *(31)* whose migratory abilities pave the way for ductal elongation. Likewise, in a time when many differentiated luminal cells are required (during pregnancy and lactation), a shift towards the production of luminal cells could be induced. The concept of a controlled balance between luminal and myoepithelial commitment has been entertained for many years by Rudland and colleagues (reviewed in *13)*.

FUTURE DIRECTIONS

Taken together, current findings suggest a common origin of HMECs and most presently detectable CFCs from a more primitive and distinct bipotent MaSC with extensive regenerative ability. Preliminary experiments in which single cell suspensions of normal HMECs have been placed in collagen gels under the kidney capsule of immunodeficient NOD/SCID mice, as first described using tissue fragments *(81)*, may now allow such a cell to be identified and characterized. Figure 3 shows an example of the type of regenerated normal human mammary cell structures that can be obtained using this approach. Future experiments using this methodology should help to answer many of the questions raised in this chapter and set the stage for understanding the relationships between primitive normal HMECs and their various malignant ($EpCAM^+/CD24^-/CD44^+$) counterparts *(82–84)*.

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