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Efficient immortalization of luminal epithelial cells from human mammary gland by introduction of simian virus 40 large tumor antigen with a recombinant retrovirus

(milk epithelial cells/*ras* gene)

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ABSTRACT **When defined in terms of markers for normal cell lineages, most invasive breast cancer cells correspond to the phenotype of the common luminal epithelial cell found in the terminal ductal lobular units. Luminal epithelial cells cultured from milk, which have limited proliferative potential, have now been immortalized by introducing the gene encoding simian virus 40 large tumor (T) antigen. Infection with a recombinant retrovirus proved to be 50–100 times more efficient than calcium phosphate transfection, and of the 17 cell lines isolated, only 5 passed through a crisis period as characterized by cessation of growth. When characterized by immunohistochemical staining with monoclonal antibodies, 14 lines showed features of luminal epithelial cells and of these, 7 resembled the common luminal epithelial cell type in the profile of keratins expressed. These cells express keratins 7, 8, 18, and 19 homogeneously and do not express keratin 14 or vimentin; a polymorphic epithelial mucin produced *in vivo* by luminal cells is expressed heterogeneously and the pattern of fibronectin staining is punctate. Although the cell lines have a reduced requirement for added growth factors, they do not grow in agar or produce tumors in the *nude* mouse. When the v-Ha-*ras* oncogene was introduced into two of the cell lines by using a recombinant retrovirus, most of the selected clones senesced, but one entered crisis and emerged after 3 months as a tumorigenic cell line.**

In the development of cancer, the changes which occur and eventually induce malignancy are expressed on the background of an individual cell phenotype, and specific features of this phenotype may play a role in the progression to invasive cancer. In attempting to study carcinogenesis *in vitro* it is therefore important to work with the appropriate cells from the tissue in which a cancer develops *in vivo*. Breast carcinomas develop from epithelial cells in the mammary gland. When the phenotype of the invasive cell is defined in terms of markers relating to the normal cell lineages, it is most frequently found to correspond to that of the luminal epithelial cell found in the terminal ductal lobular unit (TDLU) (1, 2). For *in vitro* studies on carcinogenesis in the human mammary gland, it is therefore crucial to be able to culture these luminal cells.

Human milk represents a unique source of differentiated luminal epithelial cells uncontaminated by fibroblasts, and relatively homogeneous cultures of the epithelial cells can be obtained from this source (3–5). The cells, however, have a short life-span *in vitro*, limiting their use. On the other hand, the long-term cultures and immortal lines developed from reduction mammoplasty tissue do not exhibit the phenotype of the luminal cell (6, 7). In an attempt to extend the proliferative potential of the cells cultured from milk we have introduced oncogenes that have been reported to immortalize other cell types. Here we report the development of nontumorigenic immortal cell lines from cells in the luminal cell lineage which correspond in their phenotype to the luminal cells of the TDLU. These lines represent a unique resource for studying carcinogenesis in the mammary gland.

MATERIALS AND METHODS

Cell Culture and Introduction of Oncogenes. *Media.* Primary cells from samples of human early lactation milk were grown in medium RPMI 1640 supplemented with 10% fetal calf serum (GIBCO), 10% human serum (Flow Laboratories), bovine insulin (Sigma) at 10 μg/ml, hydrocortisone (Sigma) at 5 μg/ml, cholera toxin (Schwarz/Mann) at 50 ng/ml, and antibiotics (5). Epithelial cell strains and lines derived from milk cultures via transfection or retroviral infection were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum, insulin at 10 μg/ml, and hydrocortisone at 5 μg/ml (DMEM-SIH). NIH 3T3, ψ-2 (8), and PA317 (9) cell lines were cultured in DMEM plus 10% newborn calf serum (GIBCO).

Calcium phosphate-mediated transfection. Primary human milk cells from subconfluent cultures (2–3 weeks *in vitro*) were trypsinized, washed in serum-containing medium, and replated at 3–5 × 10^5 cells in DMEM-SIH. Two to 3 hr later the cells were transfected with 1 μg of simian virus 40 (SV40) DNA (BRL) and 10 μg of calf thymus carrier DNA coprecipitated with calcium phosphate at pH 7.05 (10), carrier DNA alone being used for the control dishes. After 5 hr at 37°C, dishes were rinsed and cells were re-fed with warm DMEM-SIH. Any cells that had not attached were pelleted and added back to the original dish. Medium was changed every 3–4 days, until after 3–6 weeks colonies could be ring-cloned and expanded.

Retroviral vectors and infection procedure. Recombinant viruses based on ZipneoSV(X) (11) were developed by inserting the SV40 large tumor (T) antigen gene (12), the adenovirus early region IA protein gene (13), or v-Ha-*ras* (14) into the *Bam*HI site. Dok-v-*myc* (15) was used for introduction of the *myc* oncogene. pZiprashygro was derived from

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Abbreviations: TDLU, terminal ductal lobular unit; SV40, simian virus 40; T antigen, large tumor antigen; PEM, polymorphic epithelial mucin.

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pZiprasmyc (16) by exchanging the *Bgl* II fragment including the v-*myc* gene with a *Bgl* II fragment including the hygromycin-resistance gene *hph* (15). Ziphygro was derived from the pZiprashygro virus by deleting a 750-base-pair (bp) *Bam*HI fragment containing the coding sequences of v-Ha-*ras* (E. M. Shaufer and H. Land, personal communication). Ecotropic recombinant retroviruses produced from ψ-2 cells were used to infect PA137 cells to produce amphotropic virus. Titers (1×10^3 to 2×10^4 colony-forming units/ml) were one to two orders of magnitude lower than the corresponding ecotropic viral stocks, that of the ZipneoSV virus carrying the SV40 T antigen gene being the highest.

Subconfluent primary milk cultures were trypsinized and replated as described above and infected with supernatants containing amphotropic virus for 4 hr in the presence of Polybrene (Sigma) at 8 μg/ml; 3–5 days later RPMI-SIH containing G418 (GIBCO) at 800 μg/ml or hygromycin at 400 μg/ml was added. Control dishes were infected with ZipneoSV(X) or Ziphygro. Resistant colonies were either fixed and stained for efficiency estimation or ring-cloned for expansion and analysis of individual strains. These experiments have been carried out in accordance with the British Advisory Committee for Genetic Manipulation/Health and Safety Executive guidelines, note 5.

Immunofluorescence and Antibodies. Methanol/acetone-fixed cultures were stained by the indirect immunofluorescence technique as previously described (1). The antibodies used were HMFG-1 and HMFG-2 directed to polymorphic epithelial mucin (PEM) (17), BA16 to keratin 19 (1), C04 to keratin 18 (18), LL001 to keratin 14 (2), M20 to keratin 8 (19), RCK105 to keratin 7 (20), DP15 to desmoplakin (21), FN-3 to fibronectin (22), PAb 419 and PAb 423 to SV40 T antigen (23), PAb 421 to p53 (23), and V9 to vimentin (Boehringer).

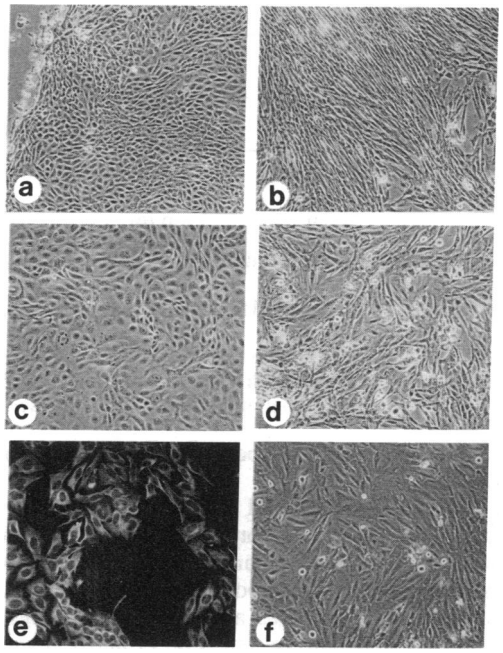

FIG. 1. Morphology of cells in milk cultures (*a*, *b*) and in SV40 transformants (*c–f*). (*a*) Epithelial colony phenotype seen in primary milk cultures. (*b*) Elongated cell type seen when milks are passaged. (*c*) Cell line MTSV1-4 passage 4, showing A phenotype. (*d*) Senescing cell strain MRSV-5.1 passage 5, showing C phenotype. (*e*) Cell line MRSV-1.2 showing A/B phenotype at passage 6 stained with antibody to vimentin to show heterogeneity. (*f*) Cell line MRSV-3.3 showing B phenotype at passage 30. (×40.) See Table 1 for description of phenotype.

RESULTS

Phenotype of Cells Cultured from Milk. Clumps of epithelial cells found in early lactation and postweaning samples of milk can be cultured with the milk macrophages, which act as feeders (4, 5). The epithelial cells proliferate, displace the macrophages, and give rise to compact colonies where the cells may be cuboidal as seen in Fig. 1*a* or more elongated. The cells in these colonies have been shown to have the phenotype of the luminal epithelial cells; they express keratins 7, 8, and 18 homogeneously and keratin 19 heterogeneously. Approximately 30% of the colonies express keratin 14, which appears to be induced by culture in some luminal cells (2). The cells also express in a heterogeneous fashion a PEM expressed by the luminal cells *in vivo*. Fibronectin is seen as a punctate surface pattern rather than as the extracellular fibrous network typical of mesenchymal cells (24).

In addition to the compact epithelial colonies, an "open" type of colony is seen in some primary cultures, where moderately elongated cells grow as a group but unattached to each other (4). These cells have a less-well-developed keratin network and a high proliferative potential; there is some evidence that they are precursors of the colonies of cuboidal cells mentioned above (25). Finally, if milk cells are passaged, a very elongated cell can emerge (see Fig. 1*b*). These "late" milk cells express vimentin and an extracellular fibronectin network, but they can be distinguished from breast fibroblasts on the basis of their pattern of junctional communications (26). It is likely that they originate from a cell type unrelated to the luminal cell (e.g., myoepithelial or myoblast), which may be found in small numbers in milk.

Isolation of Cell Lines and Strains from Milk Epithelial Cells. In an attempt to immortalize the milk epithelial cells, four different oncogenes were tested by calcium phosphate transfection and/or by retrovirus infection. In several experiments, using early region IA, v-*myc*, H-*ras*, and SV40 T antigen genes, the only oncogene found to induce an extended life-span was SV40 T antigen. More clones appeared after retrovirus infection (30–100 clones per dish) than after transfection (1–2 clones per dish). Accordingly, clones with an extended life-span were isolated from one transfection

Table 1. Phenotypes of cell strains with extended life-span isolated from milk epithelial cells by introduction of SV40 T antigen

Phenotype	Expression of protein				Pattern of fibronectin staining
	Desmoplakin	Vimentin	Keratins	PEM	
A	+	−	Basket-like	Strongly heterogeneously positive	Punctate
A/B*	±	±	Basket-like and irregular	Heterogeneously positive	Punctate and short fibers
C	−	+	Irregular pattern	Weak/negative	Fibers

Phenotypes A, A/B, and C are shown in Fig. 1 *c*, *e*, and *d*, respectively.
*Mixture of cells showing the A phenotype and cells expressing vimentin, short fibers of fibronectin, little or no desmoplakin, and an irregular pattern of keratin filaments (B phenotype).

Table 2. Crisis and emergence of immortal cell lines

Phenotype	No. of colonies with extended life-span	No. of strains showing crisis*	Total no. of immortal cell lines emerging
A†	17	8 (4)	13
A/B‡	4	1 (0)	4
C	9	9 (9)	0

*Number of cell strains that did not survive crisis is given in parentheses.
†After crisis, MRSV-4.3 adopted features of cells of the B phenotype (see Table 3).
‡MRSV-1.2 and MRSV-2.1 were later cloned and a cell line with the A phenotype was selected. With prolonged passage, MRSV-2.3 and MRSV-3.3 showed the B phenotype homogeneously (see Table 3).

experiment and from five experiments using the recombinant retrovirus.

The cell strains developed from the selected clones were examined in early passage for their morphology and expression of epithelial specific markers (see Table 1). Two main classes were seen, corresponding in morphology to the compact epithelial colonies and the highly elongated cells seen in the late milk cultures (see Fig. 1 *c* and *d*, respectively). Only the epithelial clones went on to become immortalized lines. All 9 clones selected with the elongated morphology shown in Fig. 1*d* (phenotype C) entered crisis in early passage and did not emerge (see Table 2).

When characterized by immunohistochemical staining, most of the epithelial strains similar to the one shown in Fig. 1*c* resembled the cells in the compact colonies seen in primary milk cultures. The cells expressed organized keratin filaments leading into well-developed desmosomes, high levels of PEM, and the punctate pattern of fibronectin expression typical of milk epithelial cells; they did not express vimentin. We refer to this phenotype as the A phenotype. In four cases, however, a heterogeneity in the antigenic phenotype was seen, with some cells in the culture showing the A phenotype and others characterized by irregular keratin filaments collapsed around the nucleus and the expression of vimentin and short fibers of fibronectin (B phenotype; see Table 1). An example of this mixed A/B phenotype (line MRSV-1.2) showing heterogeneous staining with vimentin is shown in Fig. 1*e*. Twenty-one clones were selected that showed the A or A/B phenotype, and 17 went on to become cell lines, in most cases without passing through a crisis period (see Tables 2 and 3). Of these, 14 were of the A phenotype, while 3 showed the B phenotype (see legends to Tables 2 and 3 and Fig. 1*f*).

Table 3. Immunohistochemical characterization of cell lines with monoclonal antibodies

Phenotype	Cell line*	Tumor growth in *nude* mouse	Crisis	Staining with antibody to keratin† 7, 8, 18	19	14
A	Group 1‡					
	MTSV-1.4	NT	None	+	+	−
	MTSV-1.7	−	None	+	+	−
	MRSV-1.2	−	None	+	+	−
	MRSV-2.1	−	p. 6	+	+	−
	MRSV-2.2	NT	p. 12	+	+	−
	MRSV-2.4	NT	None	+	+	−
	Group 2					
	MRSV-3.1	−	None	+	±	±
	MRSV-4.1	NT	None	+	+	±
	MRSV-4.5	NT	p. 10	+	±	±
	MRSV-5.4	−	None	+	±	±
	Group 3					
	MTSV-1.5	NT	p. 5	+	−	±
	MRSV-4.2	NT	None	+	−	+
	MRSV-4.4	−	None	+	−	±
	MRSV-4.6	NT	None	+	−	±
B	Group 4					
	MRSV-2.3	NT	None	+	±	−
	MRSV-3.3	NT	None	+	−	−
	MRSV-4.3	NT	p. 18	+	−	−

NT, not tested; p., passage.
*MTSV lines were derived by transfection. MRSV lines were developed by retrovirus infection.
†For antibodies used see *Materials and Methods*. +, Homogeneously positive staining; ±, heterogeneously positive; −, negative.
‡Same phenotype as most luminal cells in TDLU and most breast cancers.

Detailed Phenotypes of Immortalized Lines. With the exception of the cell line MRSV-4.3, which changed from phenotype A to B after crisis, and the lines that were initially mixed (see Table 2), the cell lines showed the same phenotype immunohistochemically at early and late passage. The phenotypes in Table 3 were determined after the cells had been in culture for 11–13 months, and they may be considered stable features of the lines. According to their reaction with the antibodies, the cell lines can be considered to fall into four groups. Groups 1–3 all show the A phenotype, and differ only in the profile of keratins they express, while group 4 includes the three cell lines that show features of the B phenotype. Fig. 2 illustrates some of the staining patterns seen in the cell lines, and it also shows the pattern of expression of fibronectin by one of the senescing cell strains of the C phenotype.

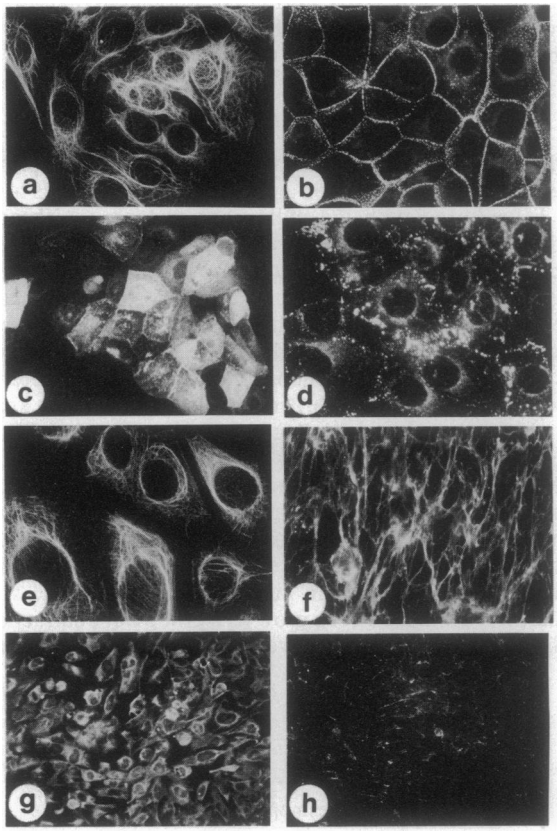

FIG. 2. Indirect immunofluorescent staining of immortal cell lines. (*a–e*) A phenotype. (*g* and *h*) B phenotype. (*f*) Senescing cell strain (MRSV-5.1) with C phenotype. Cells were fixed and stained with antibodies to the following proteins: *a*, keratin 19 (MRSV-1.2); *b*, desmoplakin (MTSV-1.7); *c*, PEM (MRSV-3.1); *d*, fibronectin (MTSV-1.4); *e*, keratin 14 (MRSV-3.1); *f*, fibronectin (MRSV-5.1 senescing cell strain); *g*, vimentin (MRSV-3.3); and *h*, fibronectin (MRSV-3.3). (*a–f* ×160, *g* and *h* ×40.)

The 7 lines in group 1 that show homogeneous expression of keratins 7, 8, 18, and 19, do not express keratin 14 or vimentin, have well-developed desmosomes, and express PEM resemble the dominant luminal epithelial cell found in the TDLU *in vivo*. Clearly the cells that were immortalized to give these lines do not respond to culture conditions by expressing keratin 14. This is an extremely important phenotype, since it corresponds to that of the majority of invasive breast cancer cells which also express keratin 19, and do not, even after years in culture, express keratin 14 (4).

Growth Properties of the Cell Lines. After transfection or retroviral infection the surviving cells were cultured in DMEM-SIH, which does not support the growth of normal milk cells. At later passages, all the lines were able to grow in the absence of hydrocortisone and insulin. None of the lines formed colonies in agar (5×10^4 cells in DMEM/10% fetal calf serum), and of the six tested for growth in *nude* mice (see Table 3) none formed tumors (10^7 cells injected subcutaneously).

When grown on plastic many of the cell lines formed dome-like structures, and in one case (MTSV-1.5) structures with lumens developed. When embedded in collagen gels some lines formed lobular structures, while others formed branching ducts. A more detailed description of the morphological structures developing in collagen gels will be given elsewhere.

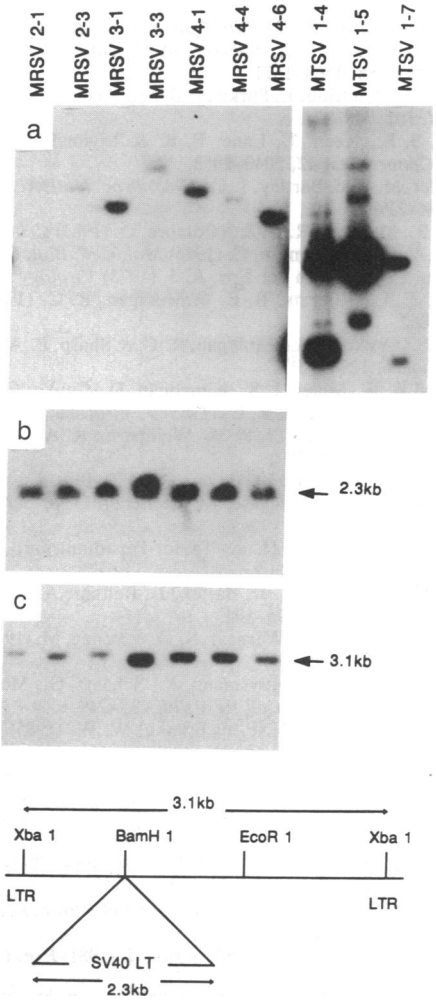

FIG. 3. Southern blots of restriction enzyme digests of DNA from immortalized lines. Samples (15 μg) of genomic DNA were digested with *Eco*RI (*a*), *Bam*HI (*b*), or *Xba* I (*c*), fractionated on 1% agarose gels, blotted onto Hybond-C membranes (Amersham) and probed with a radioactive 850-base pair *Bam*HI fragment from SV40 LT. kb, Kilobases. Restriction sites in the recombinant retrovirus are shown in the diagram. *Eco*RI cuts once within SV40 DNA, but not within the region encoding T antigen (SV40 LT). LTR, long terminal repeat. In *a*, overnight exposure of film for MTSV lanes, 7-day exposure for MRSV lanes.

Integration and Expression of SV40 T Antigen. To determine the number of integration sites, DNA from the three transfected lines (MTSV series) and from several retrovirally infected lines (MRSV series) was digested with *Eco*RI and Southern blots were probed with a fragment of the SV40 T antigen sequence. Fig. 3*a* reveals that, in contrast to the multiple integration sites seen with the transfected lines, only one is seen in the MRSV lines. Moreover, the transfected cell lines appear to contain many more copies of the SV40 T antigen genes, since the intensity of the bands in the separated digests is much higher in the MTSV lines (see legend to Fig. 3). In the case of MRSV series, the differences in the intensity of the signal seen between lanes in Fig. 3 *b* and *c* suggest that although several lines contain a single copy of the SV40 T antigen gene, amplification of the integrated DNA may have occurred in some lines, possibly by chromosomal duplication. In spite of the differences in copy number, the expression of SV40 T antigen detected by antibodies was high in all the cell lines. As expected, the expression of p53, which binds to SV40 T antigen, also increased, as detected by staining with antibodies to the amino- or carboxyl-terminal part of this molecule (data not shown).

Several cell lines were analyzed for chromosome content, and these showed a modal number in the hyperdiploid range, with a relatively broad peak; a diploid peak was also seen with the MTSV-1.7 and MRSV-4.2 lines. A high coefficient of variation, reflecting a wide spread of chromosome numbers in individual mitotic cells, is common in cells expressing an integrated SV40 T antigen gene.

Effect of v-Ha-*ras* on Immortalized Lines. Since the immortalized lines are nontumorigenic, they should be useful recipients for examining the effect of oncogenes on mammary epithelial cells. Accordingly we examined the effect of the introduction of the v-Ha-*ras* oncogene by infection with pZiprashygro retrovirus on the growth properties of two of the cell lines (MTSV-1.7 from group 1 and MRSV-3.1 from group 2). Surprisingly, most of the hygromycin-resistant clones from the pZiprashygro infection senesced quickly, but in each case one clone could be further passaged up to passage 7, at which time growth stopped, and in the case of MRSV-3.1, did not start again. After 3 months, however, the *ras*-containing clone from MTSV-1.7 resumed growth and became an immortal cell line that formed colonies in agar with an efficiency of 10% and induced tumors in the *nude* mouse with an efficiency of 40%.

DISCUSSION

Available evidence suggests that the cells which are transformed *in vivo* and which eventually become the invasive cells in breast carcinomas reside in the TDLUs (27) and belong to the luminal cell lineage (12). Since cells with this phenotype have a short life-span *in vitro*, it has been difficult to study the process of carcinogenesis in this cell type in culture. Here we describe the use of SV40 T antigen to immortalize luminal epithelial cells cultured from human milk and the characterization of 17 nontumorigenic cell lines, many of which have retained features of the common luminal epithelial cell. To our knowledge this represents the first series of cell lines definitively identified as being in the luminal cell lineage. The phenotype of cell lines in group 1 is of particular importance because this pattern of keratin expression (keratins 7, 8, 18, and 19, but not 14) is seen both in the common normal luminal epithelial cell in the TDLU and in most invasive breast cancer cells (4).

Cell lines have previously been developed from breast epithelial cells cultured from milk (6) or from reduction

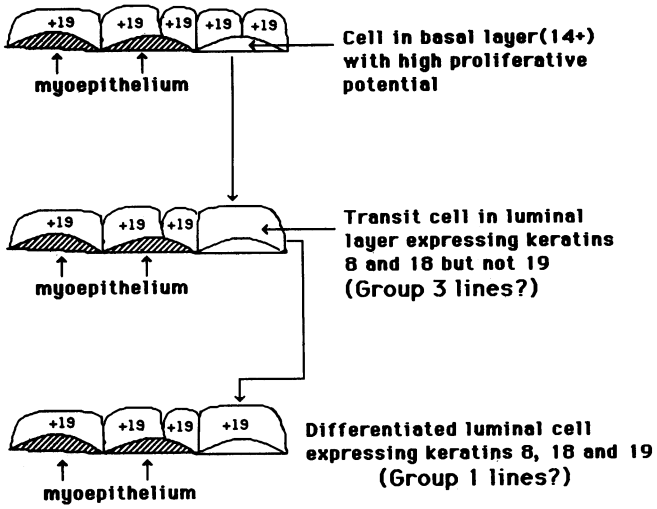

FIG. 4. Possible keratin phenotypes in the luminal cell lineage in the human mammary gland.

mammoplasty tissue (7), but the efficiency with which oncogenes were introduced (viral infection, transfection) was low, and the cell lines did not show features of the differentiated luminal cell. Infection with an SV40 T-antigen-encoding retrovirus proved to be an extremely efficient method for immortalizing the luminal epithelial cells, and it was also possible to introduce a single copy of the gene, in contrast to the complex integration pattern seen in transfected cells. With both methods, however, immortalization was achieved in most cases (12/17) without the cells passing through the "crisis" period typically seen with SV40-transformed fibroblasts. In contrast, all of the clones obtained that resembled the elongated cells found in small numbers in milk (C phenotype) entered crisis early and did not emerge to become immortal cell lines. This observation was unexpected because the elongated cells go through more divisions in culture.

On the basis of studies of the expression of keratins and differentiation antigens in the adult mammary gland (1), in the developing gland (28), and in cell cultures (2) we have suggested that the luminal epithelial cell differentiates from a cell in the basal layer (which expresses keratin 14), and that the keratin 19-negative luminal cell derives from this cell and is precursor to the 19-positive more-differentiated luminal cell (see Fig. 4). The fact that cell lines can be developed that express keratin 19 homogeneously and do not express keratin 14 suggests that these cells have passed a stage in the lineage when keratin 14 is not induced in culture. On the other hand, if the 19-negative luminal cells do indeed represent a transit cell population originating from 14-positive cells in the basal layer, it is possible that these cells could be induced to express keratin 14 in culture and would correspond to the group 3 cell lines. In the lineage scheme suggested in Fig. 4, the group 2 lines could represent a subgroup of luminal cells that have begun to express keratin 19 but in which keratin 14 expression may still be induced in culture. As yet we have not been able to relate the phenotype of the cells in group 4 (B phenotype) to the epithelial cells seen *in vivo*. They do, however, resemble the "open" type colony which can be seen in primary milk cultures (6).

Since the lines we have developed do not grow in agar or in the *nude* mouse, and they exhibit many features of the luminal epithelial cell from the human mammary gland, they represent appropriate recipients for testing the effect of other oncogenes on this cell type. When an activated v-Ha-*ras* oncogene was introduced into two cell lines it was found that most of the antibiotic-resistant clones senesced, although after a crisis period one cell line emerged that grows well in agar and in the *nude* mouse. An inhibitory effect of *ras* has been observed in REF52 cells (29) and in rat Schwann cells (30), but in these instances, SV40 T antigen expression rescued the cells from *ras*-induced growth arrest. It may be relevant that activated *ras* oncogenes have not been detected in primary breast cancers and only occasionally in metastatic lesions (31). Our results emphasize the importance of cell phenotype in defining the cellular response to a specific oncogene and support the idea that the immortalized lines described here will be valuable tools in studying carcinogenesis in the human mammary gland.

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