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Study of Rat Mammary Epithelial Stem Cells *In Vivo* and *In Vitro*

– Review –

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Abstract

Mammary epithelial cells contain a subpopulation of cells with a large proliferative potential which are responsible for the maintenance of glandular cellularity and are the progenitor cells of mammary cancer. These clonogens give rise to multicellular clonal alveolar or ductal units (AU or DU) on transplantation and hormonal stimulation. To isolate putative mammary clonogens, enzymatically monodispersed rat mammary epithelial cells from organoid cultures and from intact glands are sorted by flow cytometry according to their affinity for FITC labeled peanut lectin (PNA) and PE labeled anti-Thy-1.1 antibody (Thy-1.1) into four subpopulations : cells negative to both PNA and Thy-1.1 (B−), PNA+ cells, Thy-1.1+ cells, and cells positive to both reagents (B+). The *in vivo* transplantation assays indicate that the clonogenic fractions of PNA+ cells from out-growths of organoids in primary cultures for three days in complete hormone medium (CHM) are significantly higher than those of cells from other subpopulations derived from cultures or from intact glands. Extracellular matrix (ECM) is a complex of several proteins that regulates cell function ; its role in cell growth and differentiation and tissue-specific gene expression. It can act as a positive as well as a negative regulator of cellular differentiation depending on the cell type and the genes studied. Regulation by ECM is closely interrelated with the action of other regulators of cellular function, such as growth factors and hormones. Matrigel supports the growth and development of several different multicellular colonies from mammary organoids and from monodispersed epithelial cells in culture. Several types of colonies are observed including stellate colonies, duct-like structures, two- and three-dimensional web structures, squamous organoids, and lobulo-duct colonies. Organoids have the greatest proliferative potential and formation of multi-cellular structures. Phase contrast micrographs demonstrate extensive intracellular lipid accumulation within the web structures and some of duct-like colonies. At the immunocytochemical and electron micrograph level, casein proteins are predominantly localized near the apical surface of the cells or in the lumen of duct-like or lobulo-duct colonies. Squamous colonies are comprised of several layers of squamous epithelium surrounding keratin pearls as is typical of squamous metaplasia (SM). All-trans retinoic acid (RA) inhibits the growth of SM. The frequency of lobulo-ductal colony formation increased with the augmentation of RA concentration in these culture conditions. The current study models could provide powerful tools not only for understanding cell growth and differentiation of epithelial cells, but also for the isolation and characterization of mammary clonogenic stem cells.

Key words : mammary epithelial stem cells, extracellular matrix, Matrigel, squamous metaplasia, retinoic acid

INTRODUCTION

It has been postulated that a small subpopulation of rat mammary epithelial cells with a large proliferative potential is responsible for the maintenance of glandular cellularity. On transplantation, these cells, termed clonogens, give rise to clonal epithelial structures from which mammary cancers can arise (1). These clonogenic cells are thus the targets for neoplastic initiation. To further define the rat mammary clonogens, the studies were undertaken in an effort to concentrate and, if possible, isolate these cells by flow cytometry techniques in conjunction with quantitative transplantation and hormonal manipulation methods *in vivo* and *in vitro*.

The term clonogen was initially defined in these laboratories as a cell present in an intact mammary gland and in an enzymatically monodispersed suspension of mammary epithelial cells which, when transplanted into a subcutaneous fat pad of a syngeneic recipient, had the capacity when hormonally stimulated to proliferate and differentiate to form a clonal

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glandular structure or alveolar unit (AU). Such AU are histologically and ultrastructurally indistinguishable from the alveolar structures found in hormonally stimulated intact glands. Moreover, three weeks after mammary cell transplantation, most AU are filled with secretion (2).

These studies are designed 1) to separate the rat mammary epithelial cell subpopulations from the intact glands and increase the clonogenic fraction in a subpopulation of epithelial cells with different cell culture techniques and immunocytochemical methodology 2) to study the growth kinetics of epithelial cell subpopulations *in vitro* 3) to determine the effect of basement membrane on the growth of monodispersed epithelial cell subpopulations in culture 4) to investigate the effects of hormonal manipulation on the growth of epithelial cell subpopulations within extracellular matrix (ECM) 5) to study the effects of retinoic acid on the growth and differentiation of mammary epithelial cells cultured in ECM.

IN VIVO TRANSPLANTATION

A clonogenic rat mammary transplantation system was developed primarily as a radiobiological and hormonal effect model of cell survival and carcinogenic initiation following irradiation (1). The procedure have been described in detail elsewhere (1,2) and are summarized in Fig. 1. The concentration of viable mammary clonogens is estimated by terminal dilution transplantation assay in which mammary alveolar units in the graft sites serve as the end points (Fig. 2A) (3,4). Monodispersed mammary cell suspensions are prepared and serially diluted for transplantation. Aliquots of the cell suspensions are inoculated in the white fat pads of syngeneic recipient rats. The recipients in these assays are grafted in a hind leg two weeks earlier with mammotropic pituitary tumors to ser-

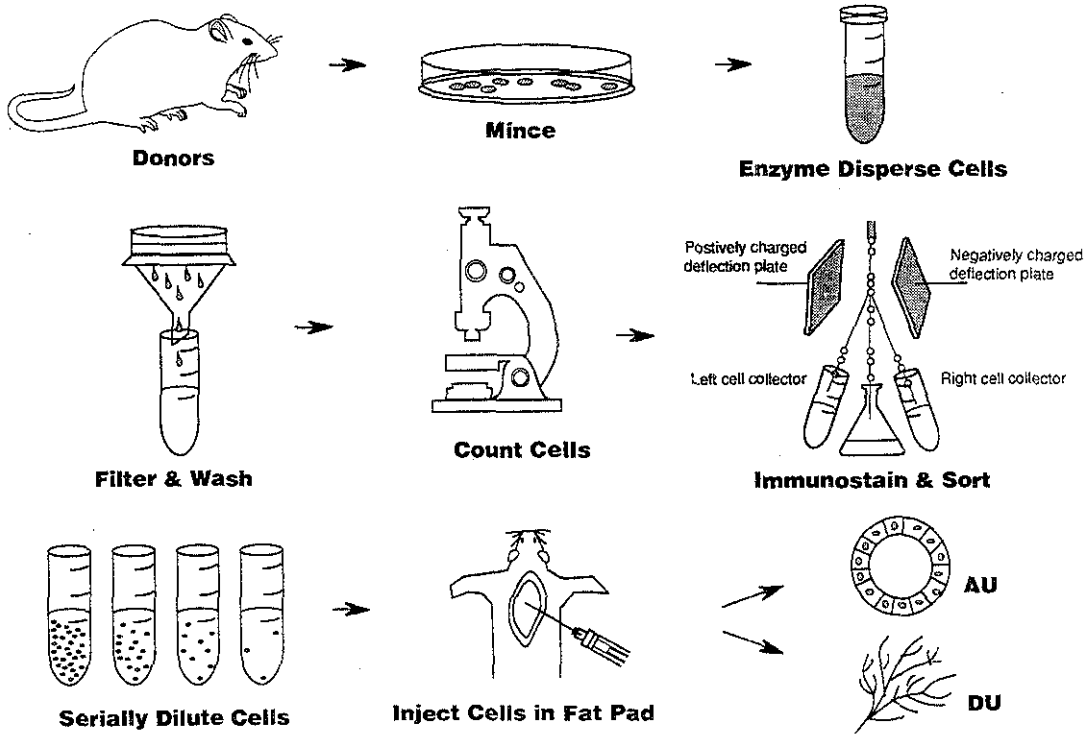

Fig. 1. Flow chart for preparation, separation, and transplantation of known number of monodispersed rat mammary cells for survival assay.

ve as internal sources of high serum prolactin (5,6). The recipient rats are killed and autopsied three weeks after mammary cell grafting. Whole mounts of the fat pad graft sites are prepared and stained for scoring of the presence or absence of AU. The fractions of graft sites with one or more AU from each concentration of graft inoculum and the mean numbers of intact cells grafted at that concentration are then analyzed according to a modification of the transplantation model of Porter *et al.* (7) using the following relationships :

$$P = 1 - e^{-KZ}$$

and :

$$\log M = \log K + S \log Z$$

where P is the probability that an AU will develop in a given graft site, M is the mean numbers of clonogens per site in that concentration of graft inoculum, K is the clonogenic fraction, Z is the mean total number of cells per site in that concentration of graft inoculum, and S is the slope of the relationship (1). S and K are estimated with the aid of a maximum likelihood iterative procedure.

AD50 (alveolar dose 50%) values, that is, the mean numbers of cells per graft site required to produce at least one AU in 50% of the graft sites, are then calculated (1,8). AD50 values are inversely proportional to clonogen concentrations and are the least variable parameter. According to this model, if the structures scored as the end points are monoclonal in origin and there are no cell interactions during their formation, S will approximate 1.0, if two cells are required to form a structure, S will approximate 0.5, and if more cells are necessary, S is progressively smaller (9,10).

Evidence consistent with the monoclonal origin of AU includes the following. Firstly, morphological stu-

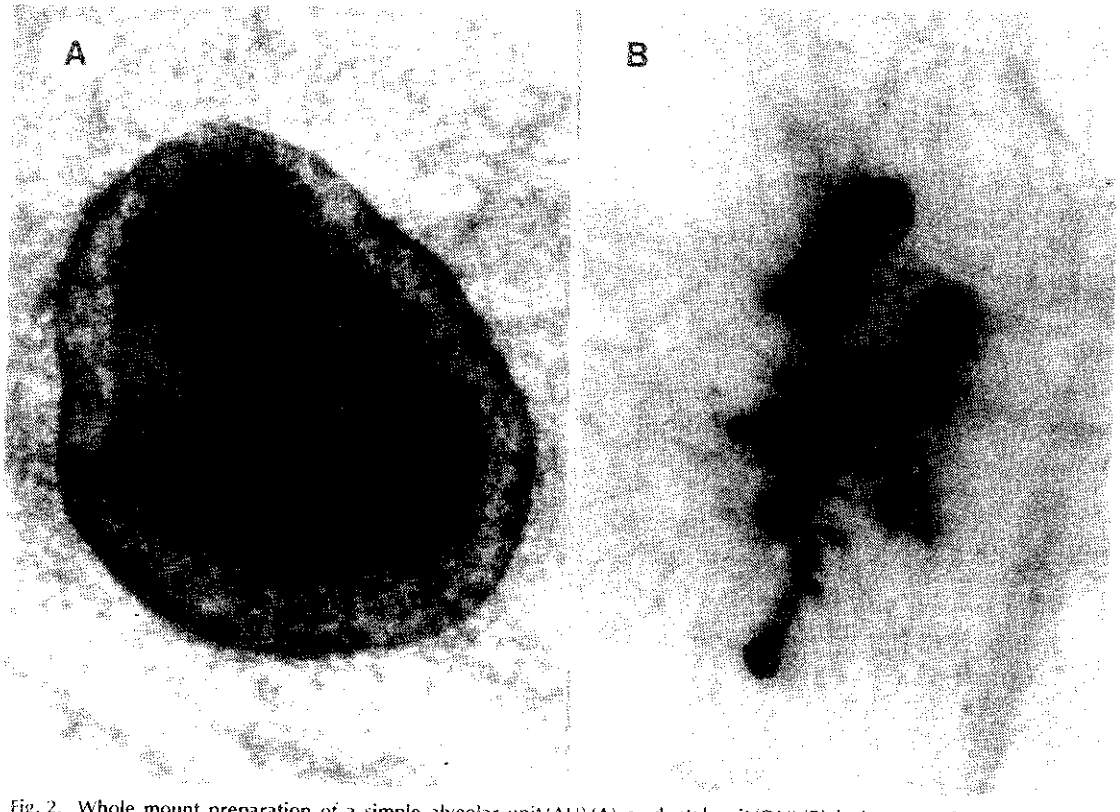

Fig. 2. **Whole mount preparation of a simple alveolar unit (AU) (A) or ductal unit (DU) (B) in interscapular fat pad that had been injected with mammary cells 3 weeks earlier.**
The recipient rat was grafted with a prolactin-secreting pituitary tumor for AU or with a prolactin-secreting pituitary tumor and adrenalectomy for DU.

dies of graft sites at intervals after mammary cell transplantation and hormonal stimulation by prolactin from co-grafted secretory MtT (mammotropic pituitary tumors) reveal that within the first few days, only scattered single epithelioid cells are observed. By the third to fifth day, occasional small spheres comprised of two to eight cells are evident. With more time, multicellular spheres lined with secretory epithelium surrounded by myoepithelial cells and with secretion filled lumina are found (9). Secondly, the fraction of transplant sites with one or more AU is quantitatively related to the number of morphologically intact cells inoculated ; the cell dose-AU response data are well fit by the single cell origin model of Porter *et al.* (7). In several tens of AD50 assays performed in our laboratories during more than a decade by several different individuals, S has routinely approximated 1.0, usually falling within the range of 0.9 to 1.1 and including 1.0 within its 95% confidence limits (4). Thirdly, the radiation dose-clonogen survival curves based on AD50 assays are characterized by an initial shoulder at low radiation doses followed by exponentially decreasing survival with increasing dose as are comparable single cell survival curves measured by clonal colony formation in culture (1,11). Lastly, studies of the physiological control of clonogen numbers in mammary glands *in situ* show that the augmentation of clonogen number is not dependent upon the number of total cells in the gland (3). AD50 assays revealed that the total number of clonogens in the mammary glands of rats with elevated prolactin did not increase despite a marked increase in total recoverable cells. However, in rats with high prolactin and glucocorticoid deficiency, the clonogen population increased more than five fold, with about the same increase in total recoverable cells.

Another type of structure, DU or ductal units, have recently been recognized (Fig. 2B) (4,12,13). DU are multibranched and non-secretory structures. DU develop from cells grafted in adrenalectomized (Adx)/ ovariectomized (Ovx) and MtT grafted recipient rats in response to high prolactin and/or estrogen. Formation of DU is further promoted by the addition of progesterone. DD50 (ductal dose 50%) values are calculated in the same fashion as AD50 values. The current studies suggest that DU are also of clonal origin. The data, so far, are consistent with the hypothesis that ductal and alveolar development are alternative pathways of differentiation by the progeny of the same sub-population of clonogenic mammary cells.

Although the evidence from a variety of experiments with mammary cells is most consistent with the clonogen hypothesis, mammary clonogens have not been isolated or morphologically defined. Attempts to characterize the developmental potential of clonogens and the effects of various external stimuli upon them have been made more difficult by this inability to identify and isolate the relevant cells. We know of no immunological reagents that react specifically with clonogens.

We have tested several different cell preparation methods (1,14) with the aim of concentrating the clonogenic cells. Cells recovered from RMEC cultured in serum-containing medium were most efficient in forming AU. However, the further enrichment of the clonogenic fraction was made difficult by the heterogeneity of the preparation from intact glands or primary cultures.

MAMMARY EPITHELIAL CELLS AND ITS MARKERS

The rat mammary gland contains more than one functional type of differentiated epithelial cell and has been postulated to contain pluripotent self renewing stem cells (15,16). The gland of the adult female rat consists of a system of branching ducts terminating in endbuds, lobules or differentiated alveoli embedded in a fatty stroma (17). The mammary ducts are composed of one or more layers of cuboidal epithelial cells bordering a lumen that is continuous throughout the gland. The epithelial cells are surrounded by a layer of elongated, myoepithelial cells (18,19). These two fully differentiated cell types have been distinguished by their characteristic ultrastructural morphologies. The ductal epithelial cells possess apical microvilli and specialized junctional complexes with associated desmosomes, whereas the myoepithelial cells possess smooth muscle-like myofilaments with

pinocytotic vesicles and a basement membrane at their ablumenal surfaces. A third functionally differentiated cell type, the secretory cells, line the alveolar lumena (20).

Recently, immunocytochemical stains have been used to distinguish the ductal epithelial, myoepithelial, and alveolar epithelial cell types in the rat. Peanut agglutinin (PNA) from *Arachis hypogaea* (peanut) binds to oligosaccharides containing the terminal sequence β-D-galactosyl-(1-3)-N-acetyl-D-galactosamine (21). The PNA has been described as a binding protein to normal human breast epithelium as well as many human carcinomas and as a marker for breast epithelial cell differentiation (22-24). PNA also stains most alveolar epithelial cells, as well as the luminal alveolar cells of rat (25,26).

Thy-1 was originally described as a cell surface alloantigen of mouse thymocytes (27). It is a small glycoprotein (MW : 17,500~25,000D) found at the surfaces of cells (28). In the mouse, it is encoded by a gene that maps on chromosome 9 (29) and the antigen has two allelic forms, Thy-1.1 and Thy-1.2. However, the rat has only Thy-1.1 (31). The Thy-1 has been identified in the adult brain (28), rat thymus (31), mammary gland (32), blood vessels, kidney collecting tubules, and certain connective tissues (33). In the mouse mammary gland, Thy-1 antigen was described as a cell-surface antigen on normal mammary gland and mammary tumor cells (32). In the rat, it was also described as a differentiation marker of potential rat mammary myoepithelial cells *in vitro* (34). It is present on and immediately adjacent to the myoepithelial cells of ducts and alveoli (35) and on the lamina densa and not on the lamina lucida (36). Myoepithelial-like cell line, Rama 29, contains 14~18 times more Thy-1 mRNA than the epithelial cell line Rama 25 (37).

MAMMARY EPITHELIAL CELL SUBPOPULATIONS

Although mammary stem-like cells have been described by several authors (16,38-41), such cells have not been concentrated from intact mammary glands for study *in vitro* and *in vivo*. Flow sorting has been used in a few studies of mammary epithelial tissues *in vitro*, including both human (42,43) and rat mammary parenchyma (44). In the current studies, flow sorting made it possible to test the clonal growth potential of sorted subpopulations of cells *in vivo* with the immunocytochemical cell surface markers peanut agglutinin (PNA) labeled with FITC and phycoerythrin (PE)-conjugated anti-Thy-1.1 monoclonal antibody and flow cytometry.

To isolate putative mammary clonogens, enzymatically monodispersed rat mammary epithelial cells from organoid cultures and from intact glands are sorted by flow cytometry according to their affinity for FITC labeled peanut lectin (PNA) and PE labeled anti-Thy-1.1 antibody (Thy-1.1) into four subpopulations : cells negative to both PNA and Thy-1.1 (B-), PNA+ cells, Thy-1.1+ cells, and cells positive to both reagents (B+) (Fig. 3) (45). With the same procedure, four different subpopulations from rat mammary tumors which developed spontaneously or were induced by carcinogens are also discerned and separated with flow cytometry (46). We have studied these subpopulations in organoid culture or in intact mammary glands and have concentrated them to perform *in vivo* transplantation assays.

The clonogen concentrations of the whole cell populations were similar through four days in culture (45). However, AD50 values began to increase after five days in culture. These results are similar to previous findings (14). Furthermore, the cells in the outgrowths around the organoids in 3 day cultures contained three times the concentration of clonogens as the organoids themselves. A two- to three-fold concentration of the assayable mammary clonogens was thus achieved by separation of out-growths from organoids. Moreover, a further two-fold increase in clonogen concentration was observed by sorting out the PNA+ cell subpopulations from the monolayer outgrowths around organoids of three day cultures. The PNA+ cell subpopulation of out-growths from three day cultures contained ~115 times the clonogen concentrations of the Thy-1.1+ subpopulation. The lowest AD50 value we have yet observed, ~82 cells per graft site, was obtained with these PNA+ cells (45). This represents about a 25-fold concentration of col-

ony forming capacity when compared to our first crude preparations, and a further three fold concentration when compared to our best previous cell preparations (14).

The B+ cell subpopulation from cultures contained one fourth to one fifth as many clonogen as the PNA+ subpopulation, but still ~27 times more clonogens than the Thy-1.1+ cell subpopulation. Although B+ cells have both cell markers on their surfaces, the AD50 assay results imply that B+ cells are more similar to PNA+ cells than to Thy-1.1+ cells in this assay system. Cells in the PNA+ subpopulation have the highest potential to give rise alveolar units in the hyperprolactinemic recipient rats suggesting that the PNA+ cell population contains the majority of multipotent mammary stem cells. The characteristics of low electron density of heterochromatin in the nuclei and secretory vesicles in the cytoplasm of most PNA+ cells suggest that they had been stimulated to differentiate by the mammotropic hormones in the medium. It seems unlikely that such differentiated cells are highly clonogenic. It may will be that the clonogens are a less differentiated subfraction of the PNA+ cells that also have oligosaccharides containing the terminal sequence β-D-galactosyl-(1-3)-N-acetyl-D-galactosamine on their surfaces and

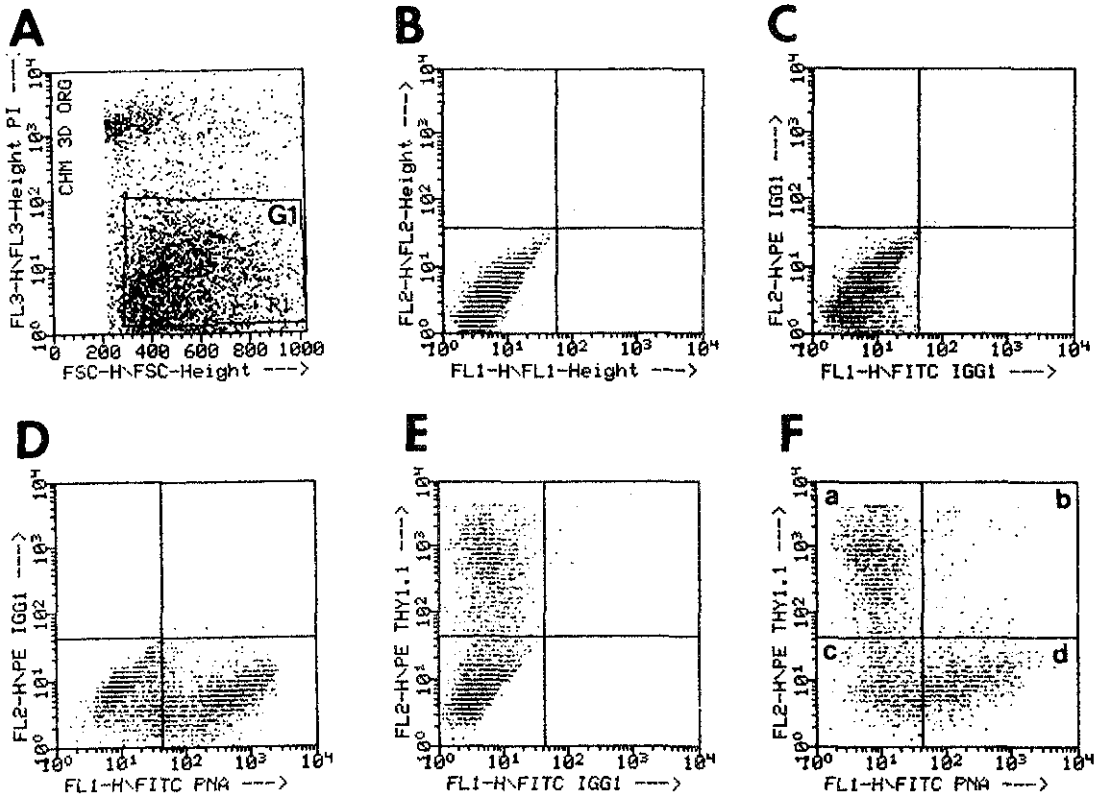

Fig. 3. Flow chart for analysis and separation of immunostained mammary epithelial cells with flow cytometry. To isolate putative mammary clonogens, enzymatically monodispersed rat mammary epithelial cells from organoid cultures and from intact glands are sorted by flow cytometry according to their affinity for FITC labeled peanut lectin (PNA) and PE labeled anti-Thy-1.1 antibody (Thy-1.1) into four subpopulations. To separate dead cells, propidium iodide-taken cells were excluded with gate setting (G1) (plate A). With the instrumental setting, natural fluorescence of unstained cells was detected (plate B). To detect the non-specific staining, samples for negative control were analyzed (plate C). Two of the positive controls stained with PNA-FITC (plate D) or with PE-Thy-1.1 (plate E) were analyzed. Finally, samples with double-stained cells with PNA-FITC and PE-Thy-1.1 were analyzed and four subpopulations were separated : cells negative to both PNA and Thy-1.1 (B-), PNA+ cells, Thy-1.1+ cells, and cells positive to both reagents (B+) (plate F).

have not yet been separable from the more differentiated cells.

Anti-Thy-1.1 antibodies bind to the cell surface Thy-1.1 antigen on normal myoepithelial cells and a myoepithelial-like cell line (16,37). Primary cultures of virgin rat mammary glands in serum medium gave three morphologically distinct major cell types : small cuboidal, cobble stone appearing epithelial cells, larger epithelioid cells, and elongated cells. The small cuboidal cells were positive to peanut lectin which binds only to alveolar epithelial cells or to ductal lining epithelial cells in the virgin rat mammary gland *in vivo* after treatment with neuraminidase (47). Electron microscopy shows that Thy-1.1+ cells have many characteristics of myoepithelial cells including prominent myofibril bundles. However, there is an overabundance of Thy-1.1+ cells after 1 or 2 weeks of culture if all of the Thy-1.1+ cells are real myoepithelial cells. Ritter and Morris (30) have demonstrated that Thy-1 is also associated with collagen-based connective tissue (mainly basement membrane) around some blood vessels. Rudland *et al.* (35) has also suggested that the cell surface antigen Thy-1 is present not only on the surfaces of myoepithelial cells *in vivo* and *in vitro* but also on the surfaces of some fibroblasts, fat cells, and cells lining small blood vessels *in vivo*. The organoids prepared from the rats possibly also contain arteriole/capillary fragments. Therefore, some of the duct-like organoids which were positive to Thy-1.1-PE might be vascular and hence mesenchymal in origin. The problem of fibroblast-like cell domination of primary mammary cultures has been noted earlier (48). Richards and Nandi (49) demonstrated that in primary cultures serum medium containing insulin, prolactin, estradiol, progesterone, and hydrocortisone support the growth of rat mammary epithelial cells as well as fibroblast-like cells in primary cultures. After 2 weeks, rat mammary epithelial cells ceased to proliferate and were eventually overgrown by fibroblast-like cells. However, the disappearance of PNA+ cells with time and the possible conversion of PNA+ to Thy-1.1+ cells in the primary cultures, the presence of prominent myofibril bundles in the cytoplasm of most of Thy-1.1+ cells, and the persistence of a low but detectable clonal growth potential in graft sites of Thy-1.1+ cells, all suggest that such cells are predominantly myoepithelium. However, the presence of tightly condensed chromatin located adjacent to the nuclear membrane suggested that they are in the early stage of apoptosis. Furthermore *Griffonia simplicifolia* agglutinin I, which specifically stains myoepithelial cells and does not stain fibroblasts *in vivo* (50), stained the large epithelioid and elongated cells but not the small cuboidal cobble stone appearing cells. We conclude that most Thy-1.1+ cells are myoepithelial in nature.

B- cells were observed in cultured colonies. However, these cells, which are both negative to PNA-FITC and anti-Thy-1.1-PE comprised a relatively small fraction, ~10 to 20%, of the whole cultured population (Fig. 2). These cells may be from the organoid itself in that most out-growth cells were positive to either PNA-FITC or anti-Thy-1.1-PE. The decrease in B- cells with culture time is consistent with this conclusion, and occurs with the progressive disorganization of the remaining organoid structures. Alternatively, cell surface marker proteins may be cleaved from some cells by trypsin.

B+ cells were a small fraction of the cell populations. Dulbecco *et al.* (15) studied the evolution of the epithelial cell types in the rat mammary gland *in vivo* with immunological markers. They suggested that the lumenal and myoepithelial cell lineages evolve separately from the stem cells because they have found no cells that appear to be transitional between the two cell types. They stained frozen sections with their different immunological markers.

It is possible that they could not detect the small subpopulation of cells positive to both markers. A possibility that can be ruled out by microscopic examination is the presence of cell clumps which contain individual PNA+ and Thy-1.1+ cells. We speculate that B+ cells are transitional cells in process of conversion from PNA+ cells to Thy-1.1+ cells or vice versa.

ROLE OF EXTRACELLULAR MATRIX

Mammary morphogenesis is a striking example of the orchestration of growth and differentiation by sys-

temic and local regulatory processes. While mammary growth clearly depends on circulating hormones for stimulation and synchronization with reproductive events, it is generally accepted that other levels of control and regulation are involved. These include interactions between epithelium and stroma, possibly involving tissue-specific growth (or inhibitory) factors, and regulatory interactions with components of the extracellular matrix (ECM) (51). ECM is composed of a variety of polysaccharides and proteins secreted by the cells. The major components of the ECM include cell adhesive or anti-adhesive molecules, such as fibronectin, vitronectin, laminin, and tenascin ; the structural components, such as collagens and elastin ; and the proteoglycans, a complex array of proteins with glycosaminoglycans side-chains (Fig. 4).

The ECM on which many of the cells are arranged is a secretory product and an active participant in regulation of their function. ECM proteins have effects on both the morphology and function of cells (52). For example, Engelbreth-Holm-Swarm (EHS) murine sarcoma-derived reconstituted basement membrane (RBM) supports the functional and morphological development of a variety of primary epithelial cells in culture (53,54) (Fig. 5). ECM has been used to support and promote mammary epithelial growth and differentiation, and even three-dimensional tissue organization and function (55-69). There was marked func-

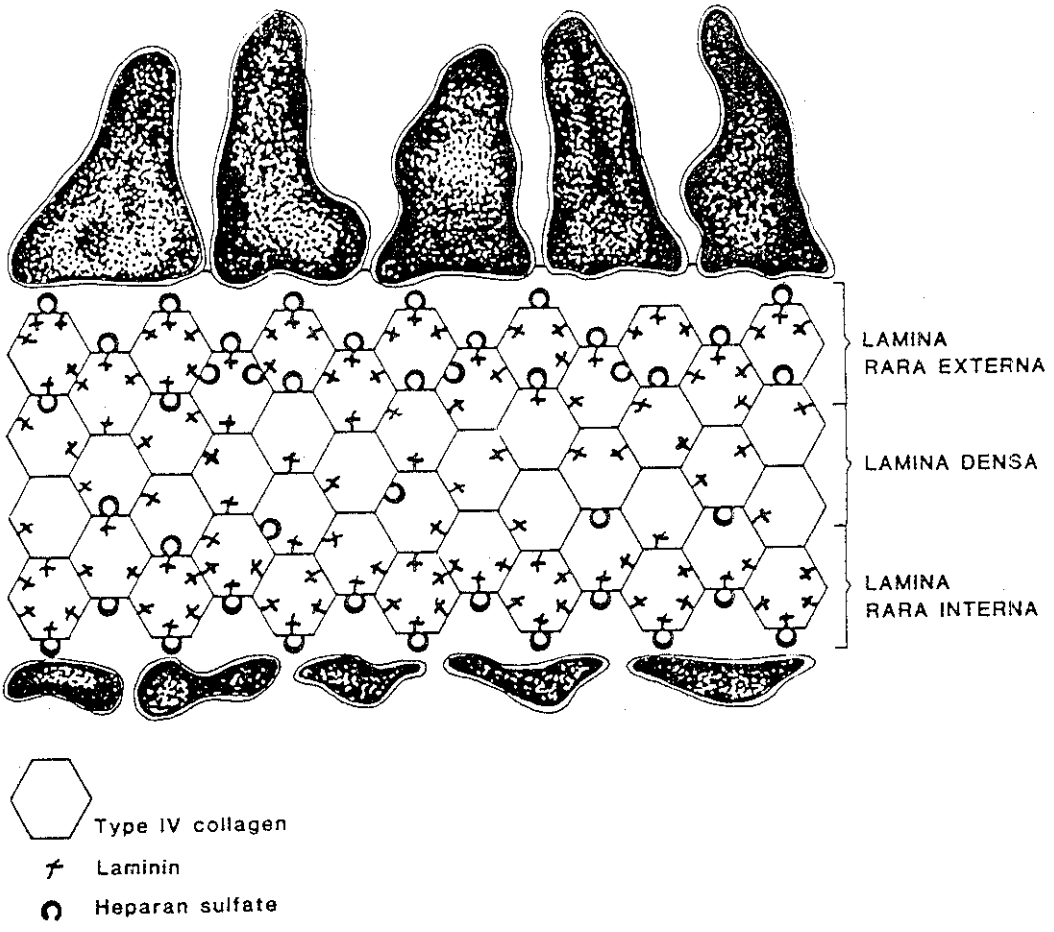

Fig. 4. Diagram showing the component of extracellular matrix proteins in basal lamina along the basal surface of epithelial membranes.
The basal lamina consists of two zones-lamina rara and lamina densa type IV collagen, laminin, and proteoglycan rich in heparan sulfate proteins are intermixed with each other in basal lamina.

tional differentiation of rat mammary epithelial cells (RMEC) cultured on basement membrane reconstituted from mammary gland (70) or the EHS tumor-derived ECM (62). Moreover, mammary epithelial cells of many species respond optimally to prolactin by producing and secreting β-casein only if the cells are placed on a ECM-like substratum or are plated in high enough density to make their own ECM (56,63,71-74). Hahm *et al.* (67,74) reported studies of RMEC in RBM derived from the EHS tumor under serum-free conditions. They demonstrated differentiation with secretion of casein and lipid as well as proliferation. RMEC growing in a ECM under defined serum-free conditions offer a unique model for investigation of the regulation of mammary development and gene expression. When mammary organoids from immature virgin rats were grown in EHS reconstituted basement membrane under defined serum-free conditions, ductal and alveolar morphogenesis occurred (68).

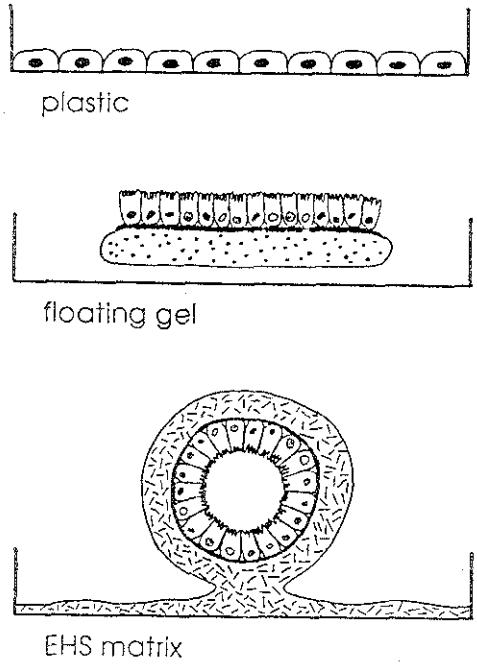

Fig. 5. **Scheme showing the relative differences in ultrastructure between cells in various culture conditions.** It is only on the floating gel or on the EHS-derived matrix that the cells are both polarized with apical microvilli and basal nuclei and are associated with a basement membrane (illustrated by the thick black line) (54).

The synthesis and secretion of milk proteins and lipids by the cultured mammary epithelial cells and organoids has been previously demonstrated under serum-free conditions (67,68,75,76). In the latter studies, tissue-specific differentiation was rapidly induced and appeared to be maintained throughout a 3-week culture period. Casein production was detected by the ELISA assay as early as day 2 and the amount of casein produced increased with time in culture. In the current studies casein was detected and located in the cultured colonies by immunocytochemical techniques confirming its production by secretory cells (77). In addition, the three-dimensional structures accumulated extensive intracellular lipid and occasionally secreted lipid globules were observed in the lumina.

Ductal or alveolar structures in mammary glands *in vivo* are comprised of well-developed secretory epithelial cells with a basal lamina which is composed of collagen IV, laminin, fibronectin, entactin, and heparan sulfate proteoglycans (78-81). Basal lamina structure has been observed in colonies cultured on a reconstituted basement membrane, on or in collagen gels in serum-containing medium (69,82,83).

In contrast, in agreement with others, mammary epithelial cells cultured in collagen gels (64) or in ECM (68) in serum-free medium failed to show basal laminal structure. The formation of basal lamina components into a visible laminal structure is influenced by several factors including serum, time in culture, and hormones (84). The possible reason for the absence of basal lamina in the serum-free medium cultures have been previously discussed (64,68). Briefly, it is possible that laminin and collagen IV are being synthesized, but secretion and/or assembly into an extracellular layer did not occur. If secreted, these proteins might be directly integrated into the preexisting gel matrix rather than forming a visible separate laminal structure. Finally, the epithelial cells may require other unknown factors for the synthesis, secretion, and/or assembly of a basal lamina which are absent from the serum-free culture conditions (77).

The development of the mammary glands is regulated by a variety of hormones and growth factors.

The complex interactions among these regulatory components are not completely understood. Moreover, the effect of these regulators on the development and functional differentiation of individual cells as well as the intercellular interactions within the mammary gland are unclear. The presence or absence of any one of these regulators, such as prolactin, insulin, hydrocortisone, estradiol, EGF, TGF-β, or growth hormone significantly affects the multicellular organization and functional development of gland. The current model system may allow systematic investigations of the effects of these regulators on *in vitro* morphogenesis of individual mammary cells and cell subpopulati ons.

SQUAMOUS METAPLASIA FORMATION

Squamous metaplasia results from aberrant differentiation of one cell phenotype into a differentiated phenotype with characteristics typical of a related tissue (85). Squamous metaplasia is observed *in vivo* in response to chronic injury or vitamin A-deficiency in several organs including the salivary and prostate glands and the respiratory and olfactory epithelia (85-90). Schaefer *et al.* (91,92) induced squamous differentiation in human breast cultures and in preneoplastic mouse mammary outgrowths by exposure to cyclic adenine nucleotide.

Squamous colonies were observed frequently among the cultures of monodispersed mixed cells, and particularly among the PNA+ cell cultures (Fig. 6A). Ip *et al.* (76) observed squamous organoids at very low frequency in optimal serum-free medium (10ng/ml EGF, etc.), but at dramatically increased levels under suboptimal conditions. They suggested that the concentration of EGF, the presence of TNF-α, and the condition (thickness) of reconstituted basement membrane affected the occurrence of squamous colonies. Low concentrations of EGF (1ng/ml or less) and/or TNF-α (0.01-100U/ml) increased the frequency of these colonies. The relatively high frequency of squamous colonies in our cultures with EGF (10ng/ml), without TNF-α, and with reconstituted basement membrane indicates that other currently unknown factors influence squamous differentiation of mammary epithelial cells. It is of importance to note that Thy-1.1+ cultures rarely gave rise to squamous colonies.

Cross sections of these colonies showed histopathologically typical squamous metaplasia (Fig. 6B). The histogenesis of squamous metaplasia is not clear. In respiratory squamous metaplasia, the most popular model is origin from pluripotent basal cells (the stem cell model) (85). The basal respiratory epithelial cells are thought to proliferate and push luminal, columnar cells upwards. These basal cells act as stem cells which can differentiate into ciliated cells, goblet cells, and secretory cells with small granules. They differentiate into squamous cells in response to vitamin A deficiency (93). The PNA+ RMEC, which are highly clonogenic *in vivo*, similarly differentiate when cultured in reconstituted basement membrane under

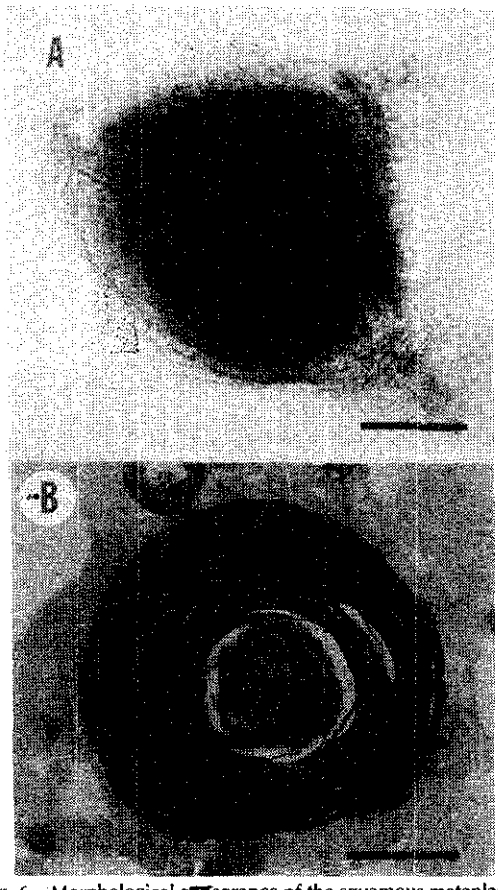

Fig. 6. Morphological appearance of the squamous metaplasia in organoids cultured in complete hormone medium. Squamous colony (A) and squamous metaplasia stained with H&E (B). Squamous structures were composed of a multilayered cells with a keratin pearl (KP) at center. Bars : 100μm.

serum-free conditions (77). Squamous metaplasia is characterized by cytokeratin expression, increased transglutaminase activity and ultimately the formation of crosslinked keratin envelopes (94-97).

Other factors may have played a role in the development of squamous metaplasia in our culture system. The process of monodispersion of the epithelial cells may have caused damage that led to metaplasia. One or more media components may have caused metaplasia, especially, in the serum-free medium which did not contain prolactin, progesterone, or estradiol. Metaplasia occurred far less frequently in cultures in CHM than in serum-free medium. It should be noted that sorting did not appear to markedly influence the development of metaplasia. PNA+ cells comprised about 50% of the mixed unsorted cells which yielded ~30% (CHM cultures) and ~55% (SFM cultures) squamous colonies ; sorted PNA+ cells yield 65~70% squamous colonies in the two culture systems.

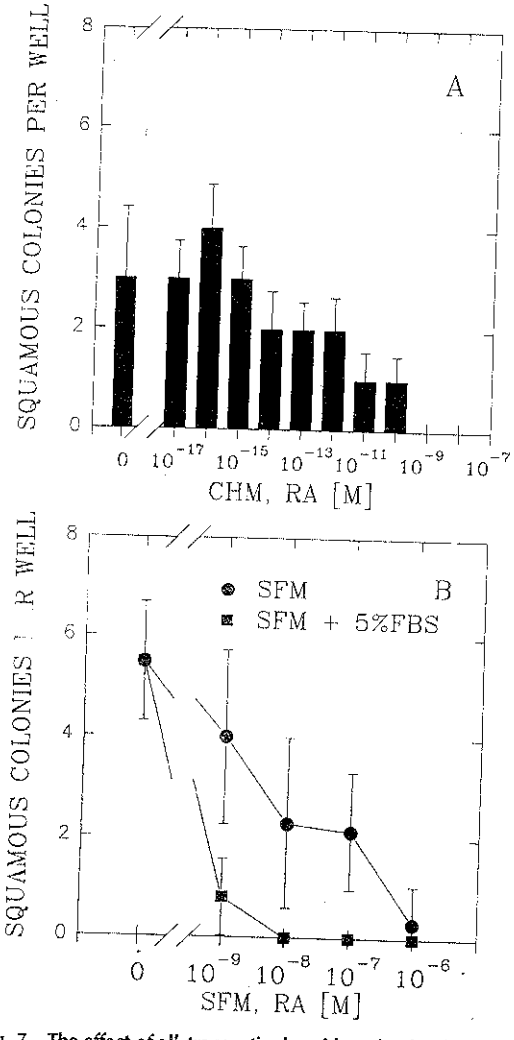

Fig. 7. The effect of all-*trans* retinoic acid on the development of squamous metaplasia cultured in CHM (A) or in SFM (B).
Each bar represents the mean±SEM of squamous colonies per well of triplicate wells. Morphology was quantitated on day 28 of culture.

Retinoic acid (RA) and related compounds play important regulatory roles in growth and differentiation of a wide variety of cell types. Indeed, retinoids are required to maintain normal differentiation and proliferation of epithelial tissues in general. The regulation of squamous differentiation by retinoids has been extensively investigated using primary tracheal epithelial cells (98) ; however, little research has been done on rat mammary glands. RA has been found to be the most potent vitamin A analogue for the reversal of squamous metaplasia in organ cultures of hamster trachea (99,100).

Organoids from donor rats initially retained their multicellular lobulo-ductal structure. During the first three days in culture, organoids appeared to gather matrix around themselves, to increase in size and to develop cellular outgrowths. By the third day, the various types of structures were observed. Squamous structures commonly had developed from tiny multicellular spheres by ten days of culture. These could be identified in undisturbed cultures by their concentric swirl appearance and rust coloration.

In section, squamous metaplasia are seen to contain keratin pearls surrounded by multi-layered basophilic cuboidal to squamous epithelial cells and are similar to squamous metaplasia that occur in many epithelial tissues *in vivo* in situations of stress (84-89). The flattened squamous layers of squamous metaplasia were positively stained with anti-pan cytokeratin antibody. The presence of squamous metaplasia suggests a) that the culture conditions, particularly serum-free conditions, are less than optimal and b) that there are significant numbers of cells capable of such differentiation in each of the cell subpopulations we have

observed in RMEC cultures.

The frequency of squamous colonies depended on the culture media. About 75% of the colonies that developed from organoids cultured in ECM in CHM were either ductal or webbed, and very few were squamous (~5%). When cultured in ECM in SFM, organoids gave rise to somewhat fewer webbed and lobulo-ductal colonies and three times more squamous colonies (~16%).

The most striking changes in response to RA are the decrease in squamous colonies and the increase in the ductal and lobulo-ductal colonies. Squamous colony formation in CHM decreased with increasing RA concentrations; development of SM was completely suppressed at 10^{-9}M RA (Fig. 7A). However, squamous colony formation from organoids cultured in SFM decreased but was not totally suppressed even at 10^{-6}M RA. To see the effect(s) of fetal bovine serum (FBS), 5% of FBS was added SFM and used for the cultures. SM development was completely inhibited at 10^{-8}M of RA (Fig. 7B). The numbers of ductal and lobulo-ductal colonies increased with increasing RA concentrations in both media; however, the frequencies of stellate and webbed colonies were not significantly changed.

Commercial fetal bovine serum contains ~190ng retinol per ml or about 10^{-6}M. As retinol would be expected to be metabolized to RA by the cells, it is likely to have contributed to the effects of the RA supplementation in the current studies. Ip *et al.* (76) have observed squamous colonies at very low frequency in rat mammary organoid cultures in growth factors-optimized serum-free medium; squamous colony frequencies were dramatically greater in suboptimal conditions. These investigators suggested that the concentration of EGF, the presence of TNF-α, and the condition (thickness) of ECM affected squamous metaplasia formation. Recently, Wada *et al.*(101) observed that phorbol 12-myristate 13-acetate completely suppressed the development of the squamous-like colonies in rat mammary organoid cultures; squamous metaplasia were frequently observed under conditions of limiting EGF concentrations. Schaefer *et al.*-(91,92) observed a similar suppressive effect on squamous metaplasia formation in mouse mammary organ cultures by phorbol ester or retinoids. Thus to completely suppress squamous metaplasia formation, mammary organoids require factors other than RA which are not present in ECM in SFM.

The development of the mammary glands and the differentiation of mammary epithelial cells are regulated by a variety of hormones and growth factors. The complex interactions among these regulatory components are not completely understood. Moreover, the effect of these regulators on the development and functional differentiation of individual cells as well as the intercellular interactions within the mammary gland are unclear. The current model system may allow systematic investigations of the effects of these regulators on *in vitro* morphogenesis of mammary epithelial cells and on mechanisms of aberrant differentiation such as squamous metaplasia formation, gene expression, and neoplastic transformation.

CONCLUSION

The current results demonstrate that the PNA+ cell subpopulation contains the most clonogenic cells. Thy-1.1+ cells appear to be terminally differentiated ; they may be primarily myoepithelial cells. B+ cells may be a transitional from one type of cell to another. No differences in morphology between AU that originated from the mixed cells of organoid cultures and AU from sorted RMEC subpopulations are detected.

Mammary organoids from virgin female rat mammary glands underwent morphogenetic development when cultured in ECM either under complete hormone medium. With time in culture, the pattern of organoid morphology changed from simple, immature mammary organoids to higher order morphologic forms. Most of the cultured organoids and monodispersed cells were rapidly induced to develop into the more complex stellate, ductal, webbed, squamous, or lobulo-ductal colonies. Ductal, lobulo-ductal, and some of squamous colonies were composed of well-polarized cells in arrangements similar to the alveolar and ductal epithelial structures of the mammary glands seen *in vivo*. Some of the cells displayed the ultrastructural characteristics indicative of fully functional secretory epithelia.

Extracellular matrix culture system may serve as a unique and useful model for investigation of the regulation of branching morphogenesis, parenchymal cell development, mammary epithelial cell differentiation, gene expression, and neoplastic transformation.

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생체 및 시험관에서 유선 상피 모세포의 분리와 동정

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요 약

유선에 존재하는 유선 상피 모세포(mammary epithelial stem cells)의 존재 증거, 정상 조직에서 이들의 역할, flow cytometry 및 면역 염색법에 의한 세포 분리, 세포 기질 단백질을 이용한 삼차원적 세포 배양에서의 증식 등을 요약한다. 유선의 실질 조직에 상피 모세포가 존재한다는 것은 여러 형태의 이식 실험에서 설명되었고 또 모세포의 표현형적 특징들은 여러 가지의 monoclonal antibodies에 의해 논증되었다. 이들 연구의 결과들은 유선의 모세포군이 end bud와 유선의 기저층(basal layer)에 존재한다고 제시하고 있다. 이들을 분리, 동정하기 위해 FITC-PNA와 PE-Thy-1.1 항체와 같은 세포 표지자를 이용하여 유선 상피 세포를 4군으로 나눌 수 있었다. FITC-PNA에만 양성 반응을 보인 PNA+ 세포군, PE-Thy-1.1에만 양성 반응을 보인 Thy-1.1+ 세포군, 이들 두 표지자에 양성 반응을 보인 B+ 세포군, 그리고 양쪽에 음성 반응을 보인 B- 세포군이었는데 이들을 flow cytometry로 분리하고 생체에 이식 실험을 하였을 때 PNA+ 세포군이 유선 모세포들을 가장 많이 가진 것으로 확인되었다. 그리고 유선 상피세포로 이루어진 유선 조직 절편(organoids) 이들 상피세포군을 세포외기질 단백질체인 Matrigel 내에서 배양한 결과 a) stellate, b) duct, c) web, d) squamous, e) lobulo-duct 등 5종류의 다세포 구조물이 생성됨을 확인하였다. 이들 중 편평상피화생의 구조물은 정상적인 유선 조직에서는 나타나지 않는 구조물인데 all-*trans* retinoic acid를 처리하였을 때 배지의 조성에 따라 다소 차이는 있으나 대부분 이들 편평상피화생의 생성이 억제됨을 확인하였다. 이상의 결과로 보아 본 연구에 이용된 생체 이식법 및 삼차원적 세포외기질 세포 배양법이 상피세포의 성장, 분화 및 모세포 연구에 유용하게 이용될 수 있으리라 사료된다.