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Expression and Differentiation: Organization and Environment

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13. ABSTRACT <i>(Maximum 200 words)</i> Breast cancer presumably originates in an estrogen receptor-positive (ER+), estrogen (E2)-responsive epithelial cell within organized human breast tissue. Paradoxically there is an exceedingly low level of, and few cells expressing, ER in normal breast tissue, and human breast epithelial (HBE) cell lines lack ER and do not respond to E2. Our hypothesis is that human breast parenchymal epithelial cell-cell interactions and epithelial stromal interactions are critical <i>in vitro</i> determinants of ER expression and biochemical differentiation. Our prior progress reported failure to demonstrate ER expression using established HBE cell lines organized into three-dimensional structures, conglomerates, resembling <i>in vivo</i> organization, and success developing and characterizing breast stromal cell resources, part of a submitted manuscript. We now report progress establishing and characterizing epithelial cells from reduction mammoplasty, benign and tumor breast tissues. Some, including cells from a tissue with demonstrable epithelial cell ER expression, failed to express ER under traditional culture conditions and in conglomerates and to exhibit responsiveness to estrogen growth. Importantly, epithelial cells from one reduction mammoplasty specimen do express ER under traditional culture conditions and in conglomerates. The latter provides evidence of significant progress towards our goal of endogenous ER expression in reduction mammoplasty-derived human breast epithelial cells.			
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FOREWORD

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(5) INTRODUCTION:

Subject:

This research project's subject is to examine cellular organization, focusing upon epithelial-epithelial and epithelial-stromal interactions, and environment as potential determinants of both estrogen receptor expression in, and differentiation by, human breast epithelial cells.

Purpose:

The rationale for this proposal was that it is generally assumed that breast cancer originates in estrogen receptor-positive [ER+], estrogen [E2]-responsive epithelial cells within structurally organized human breast tissue. Although this assumption is probably valid it is a paradox that there is an exceedingly low level of, and few human breast epithelial cells [HBE] expressing ER in normal breast tissue; a paradox extending to human breast epithelial cell lines [HBE-CL] that lack ER and do not respond to E2. The hypothesis this proposal tests is that human breast parenchymal epithelial cell-cell interactions, and epithelial stromal interactions including epithelial-ECM and stromal fibroblast interactions, are critical determinants of ER expression and biochemical differentiation in HBE cells and cell lines *in vitro*. Further, acting in this organized context the environment of hormones and growth factors are additional critical determinants of ER expression and differentiation.

Scope of Research:

The scope of this research is described in the experimental plan and specific aims. The experimental plan testing this hypothesis uses mortal [short-term cultures of] HBE cells and some of the MCF10 family of HBE-CL cultured in monolayer, in collagen to foster organization, in Matrigel to simulate the ECM, and in three-dimensional structures [formed in response to a shift from a low to routine Ca^{++} environment] resembling breast parenchymal organization *in vivo*. Breast stromal fibroblasts will be added to each condition because of evidence that these cells facilitate preservation of ER expression in rodent mammary epithelial cells *in vitro*.

The experimental plan has three specific aims. First, HBE and HBE-CL are to be cultured under these conditions to determine if interactions between epithelial cells, with ECM, and/or with stromal fibroblasts preserves ER expression in primary cultures and/or restores ER expression in HBE-CL, as well as functional response [increased PgR expression and cell proliferation] to E2. Second, analysis of these interactions will be undertaken to characterize them microscopically, and for the expression of molecular determinants, including cadherins and laminins, that mediate these interactions. Third, the functional status of these interactions will be examined by testing the ability of these cells to undergo biochemical differentiation, β -casein and α -lactalbumin expression, in response to their organizational status and environment of hormones and growth factors.

Background of previous work:

This background of previous work primarily focuses upon research pertaining to, and describes our experience with, the cell reagents required for this proposal.

A major focus in breast cancer research has been establishment and characterization of human breast epithelial cell lines [HBE-CL] that are "normal" in terms of not being tumorigenic; we recognize no cell line, by definition, can be "normal". The target was the breast parenchymal ductal-luminal cell, not parenchymal myoepithelial or stromal cells, because by several criteria these are the cells in which breast cancer originates. This goal has been accomplished to some, but not yet sufficient extent; e.g., our establishment of MCF-10A and other HBE-CL (1-4) along with cell line models of breast cancer development; e.g., MCF10 derivatives (4-10). All had probably expected that HBE-CL would express the estrogen receptor [ER] but invariably they lacked ER (1,3,11).

The method to propagate human breast epithelial cells from subcutaneous mastectomy and reduction mammoplasty tissues has these major features: culture in a low [0.04-0.06mM] Ca^{++} medium, passage of viable free-floating cells released from confluent monolayer cells, and non-serial passage to permit long term propagation (1,12,13). Mortal cells, using guidelines for phenotyping human breast cells (14-19), are of breast ductal/luminal epithelial origin based upon expression of cytokeratins [Ck]18 and 19 and of breast polymorphic epithelial mucin/EMA-epithelial membrane antigen/HMFG-human milk fat globule antigen/sialomucin/episialin, in contrast to myoepithelial or stromal cells. Mortal cells have a human diploid karyotype (1,20).

Mortal MCF-10M and MCF-10MS cells from one individual's subcutaneous mastectomy tissue [SCM-130] have on at least four occasions spontaneously immortalized. The MCF-10, MCF10-2, MCF10-3 and MCF10-4 cell lines do not undergo senescence upon serial passage into standard 1.05mM Ca^{++} medium (1,3,4). There are two sublines F and A of each, grown in low and standard Ca^{++} medium, respectively. The unique karyotype of each line proved that each arose from independent spontaneous immortalizations (1,20). These lines lack detectable estrogen receptor (1,3). All are human breast epithelial cell lines by the criteria noted above, however there are differences. By *in vitro* and *in vivo* criteria these cells are not tumorigenic (1,2,4).

The potential of mortal MCF-10MS and MCF-10 cells lines for organization has been examined to some extent. MCF-10A form three dimensional structures "domes", that are characteristic of rodent mammary epithelial cells in prolonged monolayer culture (1,3) [10A distributed by ATCC before 1998 had lost this capacity]; 10-2A and 10-3A form fewer and no domes, respectively (4). In collagen, 10A produced duct-like outgrowths; formation was hormone dependent, maximum duct formation occurred with hydrocortisone in the absence of EGF and insulin for cells cultured in $\leq 5\%$ equine serum, growth was disorganized in $> 5\%$ equine serum (1). MCF-10A reportedly (21) organizes in Matrigel and produces typeIV-collagen, a component of the basement membrane. Human breast epithelial cells including MCF-10MS, 10-2F and 10-3F lines when transferred from low to standard Ca^{++} medium undergo in a matter of hours aggregation into structures termed "conglomerates", indicating complex branching duct-like structures with end buds resembling acinar-like structures (4). This organization resembles the *in vivo* breast parenchymal morphology of the terminal ductal lobular unit [TDLU] (22) and of organoids produced by collagenase + hyaluronidase digestion of breast tissue used to culture breast cells (12,13).

We also have experience with culture of human breast stromal fibroblast-like cells. *In vivo* these cells have the highest amount of immunohistochemical aromatase (23), a rate-limiting enzyme that produces the estrogen precursor estrone. We examined (24) aromatase expression and regulation in cells cultured up to 9 passages from breast tumors and ipsilateral breast tissue distal to the tumor. These cells were characterized immunocytochemically as cytokeratin negative, α -smooth muscle actin and vimentin positive fibroblasts. Aromatase enzyme activity was increased over 1000-fold by treatment with dexamethasone alone or in combination with dbcAMP and phorbol ester. Aromatase mRNA by RT-PCR increased approximately 30 fold. Increased enzyme activity and mRNA accumulation were dose and time dependent. Therefore, aromatase expression occurs in and can be regulated dramatically in breast fibroblasts.

(6) BODY:

To reiterate from the prior report, the DOD, at the time the proposal was in the decision process for funding, was informed co-investigator Dr. Herbert D. Soule passed away January 2, 1997. This initially impacted this project, nevertheless substantive progress has been accomplished with Mr. Steven Santner joining this effort. The PI collaborated with Mr. Santner on the breast fibroblast aromatase experiments described above, it was Mr. Santner who cultured fibroblasts from breast tumor and ipsilateral benign breast tissue. Fortunately in the year prior to Dr. Soule's death he and Mr. Santner collaborated on the development of a new highly tumorigenic derivative of the MCF10AneoT cells, which is not part of this proposal, as well as in the culture of human breast epithelial cells. The cell culture work we have accomplished under this proposal has been done by Mr. Santner since he joined the project in October.

Results and Discussion:

A. Established Human Breast Epithelial Cell Lines: Organization, ER expression and Estrogen growth response:

To accomplish the goal of this proposal our initial efforts used established human breast epithelial cell lines (developed by Dr. Soule), the MCF-10 family cells described above, to evaluate for three-dimensional organization, expression of ER and responsiveness to estrogen for growth.

Three-Dimensional Organization of Epithelial Cells:

Our original proposal included the generation of "conglomerate" structures of certain epithelial cells; termed conglomerates to indicate the complex organization of polarized epithelial cells and lumen-resembling spaces, which were described in the Preliminary data. These were MCF10-2F and MCF10-3F cells, routinely cultured in low Ca⁺⁺ medium, that when switched to routine [high] Ca⁺⁺ medium formed complex three-dimensional structures that macroscopically and microscopically resemble the organoids characteristic of breast parenchymal tissue initially out of the host.

We initiated cell cultures to generate these conglomerates from frozen stocks of cells. Our results indicated that prolonged culture [>1 month] is required following reactivation of a frozen stock. We have succeeded in forming conglomerates from MCF10-2F and MCF10-3F. We have done these experiments with the ER+, E2-responsive human breast cancer cell line MCF7, which will form loose aggregates with less obvious morphologic organization, to provide ER+ controls for expression analyses [immunochemistry]. No difference in MCF7's ability to form these loose aggregates has been noted in three experiments comparing cells growing with E2 supplementation as compared to E2 depleted medium.

ER expression and Estrogen responsiveness:

We examined for ER expression in monolayer cultures and for E2 growth responsiveness in monolayer cultures of MCF10 and MCF10-2 cells, as well as MCF7 as a positive control for ER expression and E2 growth responsiveness. Immunocytochemical analyses to date have not detected ER in MCF-10A cells; MCF10-2A cells have less than 5% of cells staining weakly which we do not regard as a reliable positive signal. No reliable differences in growth rate of MCF-10 or MCF10-2 cells have been noted culturing in E2 supplemented media or in media with an anti-estrogen [4-hydroxy-tamoxifen or ICI 182780]. In contrast, MCF-7 cells demonstrated an approximately 4-fold increase in growth with E2 supplementation compared to E2-depleted or anti-estrogen treated cell cultures.

B. Short Term Human Breast Epithelial Cell Cultures: Tissues and Cells

Tissue Acquisition and Characterization:

Human breast tissues have been obtained through the Karmanos Cancer Institute Tissue Resources

Cores, directed by Dr. Wael Sakr. Protocols for tissue acquisition were and are approved by the IRB-Human Investigation Committee. Our protocol blinds us from any patient identifiers and clinical information but tissue histopathology is available for some specimens.

During this period the majority of specimens have been from reduction mammoplasty tissues. To date 28 tissues have been processed for cell culture including 19 reduction mammoplasty tissues, and 9 matched/paired breast tumor and ipsilateral benign breast tissues from the same 9 patients. As noted previously we have implemented routine histological characterization of the majority, and now all, tissue specimens by Mr. L. Tait. The justification for this effort is prior and current experience: three "tumors" had no identifiable tumor cells, and one benign tissue had carcinoma in situ (CIS) disqualifying cells cultured from this sample.

Cell Cultures:

As stated in the experimental plan short-term cultures of reduction mammoplasty, benign breast and breast tumor epithelial cells and fibroblasts were cell resources that must be developed to accomplish the goals of this proposal, should we be unable to demonstrate ER expression and an estrogen growth response of the established cell lines. As noted above we were unable to demonstrate ER expression and estrogen growth response in our established cell lines, therefore a substantial portion of our effort is for generation of these new cell resources. The experimental methods and procedures for culture of cells are as reported in our publications.

Cell and Cell Line Characterization:

Cell morphology is cuboidal for epithelial and elongated for fibroblasts mortal cells and cell lines. Immunocytochemical analyses have been done as proposed. Both mortal cells and cell lines are the same for the expression of epithelial and fibroblast phenotypic markers: Epithelial mortal cells are pan cytokeratin positive, whereas Fibroblast mortal cells and extended life span cells have <5% cytokeratin weakly positive cells. Vimentin is not a distinguishing phenotypic marker between cell types. Another distinguishing phenotypes is that a portion of fibroblasts express a high level of α -smooth muscle actin. These results indicate that epithelial and fibroblast mortal cell cultures and cell lines have distinguishing phenotypic properties, and that long-term cultures preserve phenotypic markers of parental mortal cells.

Therefore it is by the immunohistochemical parameters that cultured cells are defined to be epithelial or fibroblast in phenotype. It is prolonged serial passage in vitro, which for epithelial cells involves a shift from low to high calcium medium, that distinguished mortal cells from cell lines.

C. Short Term Human Breast Epithelial Cell Cultures: ER Expression and Estrogen Responsiveness:

The approach we will take in this report is to describe several properties of Human Breast Epithelial Cells for several of these we have successfully cultured for an extended time. Cultures are not always successfully established from tissues, in some cases because few cells are recovered, limited proliferative capacity, and contamination that cannot be countered.

Epithelial Cells, reduction mammoplasty-derived WS-24

Tissue Characteristics:

Reduction mammoplasty tissue with normal parenchymal morphology and fibrous stroma. No evidence of hyperplasia, atypia, CIS or invasive carcinoma cells. Characteristic Ck19+ ductal/luminal epithelial cells, myoepithelial cells, no discernable ER+ cells, significant proportion of cells with distinct nuclear PgR expression. In MCF-7 cells the expression of PgR is a response to estrogen, which here implies ER function.

Epithelial Cell Cultures and Phenotype:

Human breast epithelial cells based upon the above criteria with substantial growth as mortal cells have now been maintained over 10 months by non-serial passage in low calcium medium. In routine non-serial free-floater cell passages WS-24 form an adherent monolayer and then produce viable free-floating cells.

Immunohistochemical characterization routinely uses cells cultured onto plastic chamber slides. WS-24 cells are Human breast epithelial cells based on expression of Ck18, Ck19 and HMFG-1/human milk fat globule membrane antigen expression. WS-24 cells on chamber slides, however, do not grow as monolayer cells, either in low or high calcium medium; few cells attach and spread in low calcium, most are poorly adherent rounded cells in which it is difficult to discern between cytoplasm and nucleus. In high calcium medium more cells attach but they commonly form large aggregates which interfere with immunocytochemistry.

A limited number of fibroblasts were cultured from the same tissue.

ER and PgR Expression:

A significant proportion of cells have nuclear reactivity for the ER 1D5 antibody and PgR, although at a lower intensity than MCF-7 cells analyzed at the same time. This phenotype applies to non-serial passage low calcium medium cells and cells switched to high calcium medium immediately prior to immunocytochemistry.

Conglomerate Formation, ER and PgR Expression

Upon a switch of WS-24 epithelial cells from routine low calcium to high calcium medium the cells organize into structures, although the level of organization in these aggregates is not high. Cyst-like structures are present in a significant proportion also, and there is some indication of some terminal ductal-lobular unit like structures. As with routine cell cultures significant expression of Ck19 and HMFG-1 were detected, but no expression of alpha-smooth muscle actin was detected (the latter is an indicator of a myoepithelial phenotype among epithelial cells).

Immunochemically no PgR expression was detected but a significant, about 25%, expressed detectable ER ranging from low to high levels, that was preferentially localized in the nucleus.

These observations were with two separate preparations of mortal WS-24 cells.

Effect of Estrogen on ER Expression:

WS-24 human breast epithelial cells were cultured in routine medium and in 17beta-estradiol supplemented medium prior to conglomerate formation. Conglomerates formed from cells grown in medium not supplemented had detectable ER, as above. Conglomerates formed from cells grown in estradiol-supplemented medium had no detectable ER. These observations have been reproduced, and imply that estradiol supplementation may down regulate ER in some manner.

Therefore, WS-24 epithelial cells although difficult to culture at this point as traditional monolayer cells exhibit ER expression, which was the goal of our project. At this time we have several goals to accomplish with these cells. First, is to substantiate ER expression by RNA analysis and protein characterization by Western blotting. Second, to evaluate for a growth response. Third, we will characterize other parameters including expression of cell adhesion molecules, the modulation of ER expression by estradiol, and other studies proposed in the experimental plan. Fourth, is to establish a stable cell line that maintains ER expression and estrogen responsiveness by multiple criteria. If this last goal can be accomplished these cells could be made available to the scientific community and would likely be considered a significant scientific resource.

Epithelial cells, reduction mammoplasty-derived, WS-15 and WS-39:

Tissue Characteristics:

Reduction mammoplasty tissues with normal parenchymal morphology and fibrous stroma.

Epithelial Cell Cultures and Phenotype:

Cells proliferate in non-serial passage in low calcium medium.

Human breast epithelial cells based upon expression of specific cytokeratins and HMFG-1.

ER Expression:

Neither WS-15 nor WS-39 express detectable ER either in low calcium nor in high calcium medium.

Effect of Estrogen:

In WS-15 cells no growth response to estrogen supplementation in estrogen depleted cells was observed. WS-39 cells are being tested, which takes over one month to first adapt cells to growth under controlled estrogen conditions.

Conglomerate Formation and ER Expression:

WS-15 cells do not organize upon a switch from low to high calcium medium to form conglomerates. WS-39 cells do form conglomerate-like structures but in two separate experiments ER expression was not detected immunocytochemically, in contrast to control MCF-7 cells.

Epithelial cells, ipsilateral benign breast tissue-derived, WS-16 and WS-38:

Tissue Characteristics:

Benign breast tissues are from the ipsilateral breast as distal as possible to the breast tumor. Grossly and histopathologically these tissues had normal parenchymal morphology and fibrous stroma; no localized (carcinoma in situ) or invasive tumor cells were microscopically detectable. Immunohistochemical characterization of the original specimen from which WS-38 cells were cultured demonstrated an ER and PgR positive phenotype of a substantial proportion of breast epithelial cells.

Epithelial Cell Cultures and Phenotype:

Extensive cell proliferation occurs by non-serial passage of cells in low calcium medium. WS-16 cells have been transferred into high calcium medium for short term experiments to characterize cells but WS-16 cells do not exhibit continual proliferation in high calcium medium.

Immunocytochemical characterization of WS-16 cells in both low and high calcium medium indicates a cytokeratin positive and HMFG-1 positive phenotype consistent with human breast epithelial cells.

ER and PgR Expression:

Neither WS-16 nor WS-38 cells expressed detectable nuclear-localized ER or PgR expression cultured in low or high calcium medium.

Conglomerate Formation and ER Expression:

Both WS-16 and WS-38 epithelial cells form conglomerate-like structures upon a switch from low to high calcium medium. In neither, in at least two independent experiments with each type, has organization resulted in detectable ER expression immunohistochemically.

Fibroblasts, reduction mammoplasty, ipsilateral benign breast and breast tumor-derived:

Tissue Characteristics:

WS-14 and WS-15 fibroblasts were derived from reduction mammoplasty specimens of two different patients. These tissues had normal parenchymal and fibrous stromal morphology. WS12 fibroblasts were derived from both an ipsilateral benign breast tissue specimen and a breast tumor tissue specimen.

Fibroblast Cell Cultures and Phenotype:

Methods for culture of fibroblasts from breast tissues have been reported by us and are used for these studies. These cells have an elongated fibroblast-like morphology, in contrast to cuboidal epithelial morphology. Immunocytochemical analyses demonstrated a cytokeratin negative, vimentin positive and alpha smooth muscle actin positive phenotype indicting the fibroblast nature of these cells.

ER expression:

Estrogen receptor has not been detected immunocytochemically in these cells.

Estrogen Metabolism:

We have used these fibroblasts to examine for the transcriptional regulation of the CYP19 gene that codes for the rate limiting aromatase enzyme in estrogen synthesis by extending our previous studies (24) to include evaluation of transcription from six of the 5' alternative transcription initiation sites. The results of these studies, including the development of extended life fibroblasts by retroviral delivery of the HPV E6 and

E7 oncogenes (25), were briefly described in the last report. Our results now are under review for publication:

Pauley, R.J., S.J. Santner, R.K. Bright and R.J. Santen. Regulated CYP19 Aromatase Transcription in Breast Stromal Fibroblasts. Submitted to the Journal of Clinical Endocrinology and Metabolism.

The results demonstrate for the first time that fibroblasts from these breast tissues all exhibit regulated CYP19 transcription from alternative first exons EI.3, EI.4 and PII, the same first exons expressed in these breast tissues in vivo. The results also provide direct evidence that the phenomenon of "exon switching", the preferential use of first exon EI.4 in reduction mammoplasty tissues compared to EI.3 and PII in breast benign and tumor tissues, is not due to an intrinsic difference between fibroblasts from these tissues but can be explained by response to different transcriptional regulatory molecules among these tissues.

We have provided fibroblasts to our colleague Dr. P.V.M. Shekhar who is studying cell-cell interactions between the MCF10AT cells and vascular endothelial cells, so that three cellular components of breast tissue can be combined in vitro to test for organization among these different cell types.

(7) KEY RESEARCH ACCOMPLISHMENTS

- Development and characterization of epithelial cells from reduction mammoplasty tissues
- Development and characterization of epithelial cells from benign breast tissues
- Development and characterization of fibroblasts from reduction mammoplasty, benign and tumor breast tissues.
- Organization of breast epithelial cells into structures, conglomerates, resembling human breast organoids.
- Demonstration of WS-24 reduction mammoplasty epithelial cells to express Estrogen Receptor in routinely cultured cells and conglomerates; this is the major goal of our proposed research..
- Demonstration that breast reduction mammoplasty-, benign-, and tumor-derived fibroblasts exhibit hormonal responsiveness for control of aromatase expression, the rate limiting enzyme for estrogen synthesis

(8) REPORTABLE OUTCOMES

- Development of Immortalized Cell Lines: Although not a goal of this research, the WS-15 reduction mammoplasty-derived breast epithelial cells have exhibited extended growth in vitro consistent with immortalized cell line behavior.
- Development of Mortal Epithelial Cells: Mortal cultures of reduction mammoplasty and benign breast epithelial cells have been established but these cells have limited capacity for long term propagation compared to immortal cell lines.
- Development of Mortal Fibroblasts: Mortal cultures of reduction mammoplasty, benign and tumor breast fibroblasts have been established but these cells have limited capacity for long term propagation compared to immortal cell lines. Extended life span derivatives were also established of benign and tumor breast fibroblasts, but again these are not immortal cell lines.
- Manuscript submitted: Pauley, R.J., S.J. Santner, R.K. Bright and R.J. Santen. Regulated CYP19 Aromatase Transcription in Breast Stromal Fibroblasts. Submitted to the Journal of Clinical Endocrinology and Metabolism.

(9) CONCLUSIONS:

The Results described above provide evidence of progress in key elements including:

- [1] Generation of mortal human breast epithelial cells from fresh reduction mammoplasty tissue.
- [2] Propagation of fibroblasts from fresh reduction mammoplasty, benign breast, and breast tumor tissues.
- [3] Experiments with established human breast epithelial cell lines, MCF-10, MCF10-2 and MCF10-3, have failed in monolayer culture and in cells organized into three-dimensional structures termed conglomerates fail to detectable express the Estrogen Receptor.
- [4] Experiments with the reduction mammoplasty-derived WS-24 mortal human breast epithelial cells have provided evidence for ER expression in cells grown on plastic and in three-dimensional conglomerate-like structures. Importantly, initial evidence is presented that 17beta-estradiol diminished ER expression in these conglomerate-like structures. These results indicate substantial progress to the ultimate goal of this project, having non-transformed human breast epithelial cells that express the Estrogen Receptor.
- [5] Experiments with two other reduction mammoplasty-derived and two benign breast tissue-derived mortal human breast epithelial cells on the other hand have failed to reveal detectable ER expression, even in cells from one tissue that had detectable ER expression in substantial proportion of the benign breast epithelial cells. Among these cells that form conglomerate-like structures ER expression also was not detected.

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