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FOREWORD

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Mammary Gland Ontogeny and Neoplasia in Oxytocin Deficient Mice

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The hormonal environment in which mammary tumors develop and are maintained is important for understanding not only the progression, but also the potential strategies for treatment, of breast cancer. A possible role for endogenous oxytocin (OT) in human breast cancer has not been extensively studied. OT functions as both a hormone and neuropeptide. In the periphery, OT contracts the myoepithelial cells of the breast to elicit milk ejection during lactation and the myometrial cells of the uterus at parturition. Mammary myoepithelial cells and uterine myometrial cells express receptors for OT (OTR), which mediate its contractile effects within these cells. The OTR is a seven transmembrane domain polypeptide that belongs to the family of G protein-coupled receptors.

Exposure to stimuli that release OT provide the mammary gland with a higher than basal concentration of this hormone, which is approximately 1pg/ml in most mammals including humans. As an example, nursing by the young induces a robust release of OT, with plasma concentrations 10-50 fold greater than basal. Plasma OT concentrations are also increased above basal with vaginal distension, such as occurs during childbirth, and during estrogen dominated states such as the luteal and mid phases of the menstrual cycle, and pregnancy. A woman's cumulative exposure to OT is dependent upon the number and duration of experiences that trigger OT release. Of note is that many of the events associated with elevated levels of OT are also associated with a reduced relative risk of developing breast cancer. The consequences of variable exposure of the mammary gland to OT has not been extensively investigated. Data invoking a protective role for OT in breast cancer are preliminary but compelling. OT inhibits the proliferation of human breast cancer cell lines and the growth of mammary, but not colonic, cancers xenotransplanted into rodents.

The increase in OT concentrations during late pregnancy is also accompanied by an increase in the number of uterine myometrial OTRs. Likewise mammary myoepithelial OTRs are markedly increased during lactation. These changes in OT and its believed to result, at least in part, from the dramatic changes in estrogen and progesterone which accompany pregnancy and lactation. Within the hypothalamic paraventricular and supraoptic nuclei (PVN and SON), which are the sites of synthesis of over 95% of the OT within the central nervous system, (CNS), as well as within the uterus, an extra CNS site of OT synthesis, estrogen facilitates and progesterone inhibits OT expression

Estrogen and progesterone exert the same effects upon the OTR. Both myometrial and myoepithelial OTRs are increased by estrogen and decreased by progesterone.

Human mammary cancers which are typically of epithelial, not myoepithelial, origin also express the OTR. OTR localizes to myoepithelial cells in normal, but to epithelial-derived tumor cells, in cancerous breast tissue. The function of the OTR in human breast cancer cells is not well understood. The regulation of the OTR by estrogen and progesterone has not been well studied in cancerous human mammary epithelial cells.

A series of experiments were performed using MCF-7 and HS578T human breast cancer cell lines, and mice deficient in OT, to test the hypothesis that OT inhibits growth of breast cancer cells and that acquisition of the OTR by mammary epithelial cells accompanies malignant transformation.

PROGRESS REPORT

One hypothesis is that acquisition of the OTR by mammary epithelial cells accompanies malignant transformation. If correct, epithelial cells of normal or tumor adjacent tissue will not express OTR, but myoepithelial cells will. In contrast breast cancers which derive from epithelial cells will express OTR. Using a specific monoclonal antiserum to OTR, we performed immunohistochemistry of normal human breast tissues removed from women undergoing reduction mammoplasty, and of cancerous and tumor adjacent breast tissues removed from women at mastectomy. In **normal** mammary tissue, OTR is present in **myoepithelial** cells. In eight cases of ductal carcinoma (the most common form of epithelial mammary cancer), the **breast cancer epithelial** cells expressed OTR, **fig 1 A**, whereas in **tissue adjacent to the cancer**, OTR was in the **myoepithelial** cells, **fig 1B**. The anti OTR monoclonal antibody (O-2F8) used in these studies was obtained from Dr. Hachiro Yamanaka (Rohto Pharmaceuticals, Japan) and specifically detects OTR protein. This antiserum also stained the myometrial cells of the uterus (not shown), which are known to express OTR, verifying the specificity of the technique.

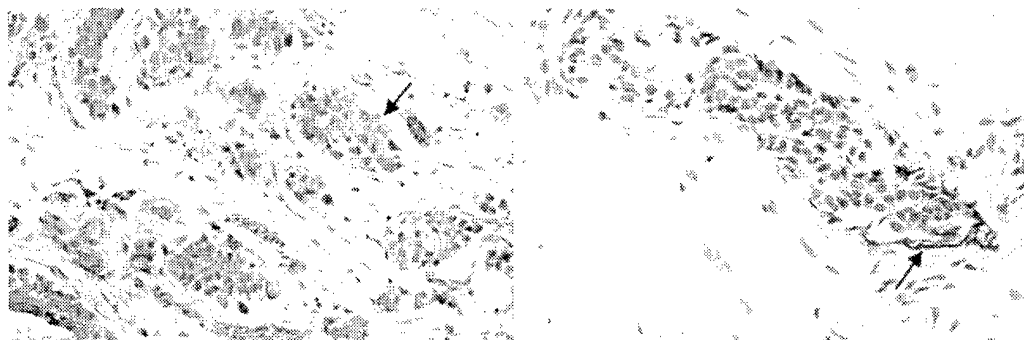


Fig1. Breast tissue removed from segmental mastectomy. The ductal carcinoma on the left stains positively with the antiserum to OTR in the epithelial cells whereas the adjacent noncancerous breast tissue from the same patient on the right has positive staining for OTR in the myoepithelial cells but no staining in the epithelial cells. 200X magnification

RNA was extracted from human tissue samples and reversely transcribed with primers for OTR. We readily detect by RT-PCR the OTR product in normal (from reduction mammoplasty) and cancerous (ductal and lobular cancers) mammary tissue samples. The mammary PCR products are the same size as the uterine OTR product. PCR yielded an amplified band of 397 bp (corresponding to bp 1215-1620 of the human OTR mRNA) **fig2**. This 397bp- OTR PCR product is also present in human breast cancer cell lines HS578T, MCF-7, and BT-20 (American Type Culture Collection, ATCC, Rockville, MD).

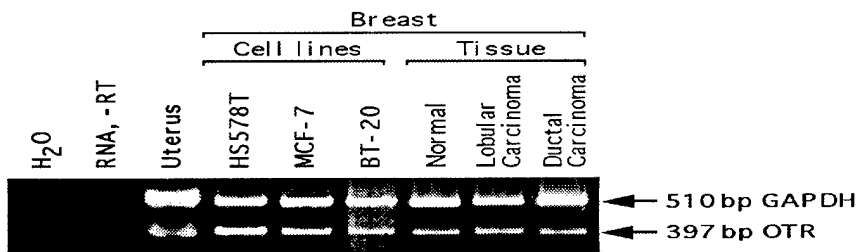


Fig 2. Samples of RNA (1mcgm) from human breast cancer cell lines (HS578T, MCF-7, BT-20), and normal and cancerous human breast tissues were reverse transcribed with OTR and GAPDH specific primers, and each transcript was amplified by PCR. A 397-bp OTR PCR product was identified in breast cells and tissues Also shown are lane 1, water; lane 2, RNA without reverse transcriptase; lane 3, human uterine RNA, a tissue known to express OTR. The normal breast tissue was obtained from a woman undergoing reduction mammoplasty.

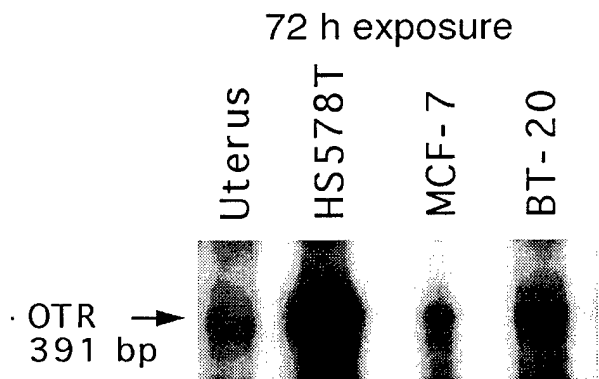


Fig 3. Solution hybridization/RNase protection assay. Each lane represents RNA (20 μ g) from human breast cancer cells and term uterine tissue. In each sample the appropriate protected band, of 391 bp, was identified.

OTR mRNA in these human breast cancer cell lines is readily measurable by solution phase RNase protection assay. The protected 391-bp fragment in these cells is also present in human uterine tissue, **fig 3**.

Because of the ease of obtaining and culturing human breast cancer cell lines, our preliminary studies of OT effects upon cell growth and regulatory studies of OTR were performed in MCF-7 and HS578T cells. We found that OT (10^{-6} to 10^{-8} M) inhibits cell growth in mammary tumor cell lines grown in serum-supplemented medium. We identified this growth inhibitory effect of OT in

MCF-7 cells, **fig 4**, and in HS578T cell, not shown. Cell counts were done with a hemocytometer and trypan blue staining was performed to verify cell viability. OT (10^{-7} to 10^{-8} M) also decreased [^3H]-thymidine incorporation into cells, **fig 5**.

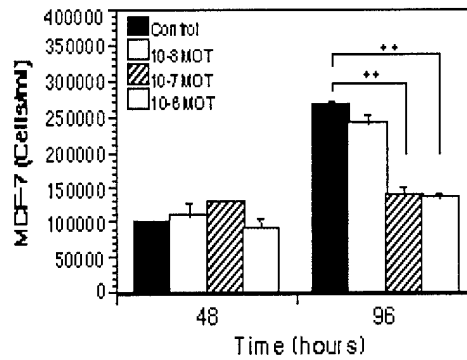


Fig 4. MCF-7 cells were grown to confluence and exposed to OT (10^{-6} to 10^{-8} M) or vehicle for 48 and 96 h. Medium supplemented with serum was changed every 24 h. The data are reported as the mean \pm SEM of triplicates at each point. Compared to vehicle-treated cells, cells treated with OT had a significantly lower rate of proliferation at 96 h. ** control vs 10^{-7} or 10^{-6} M OT, $p < 0.0001$, Fisher's PLSD.

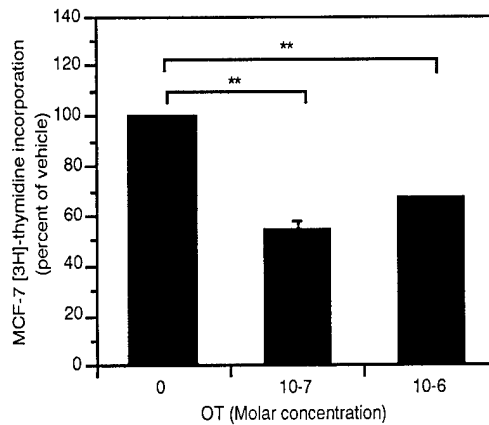
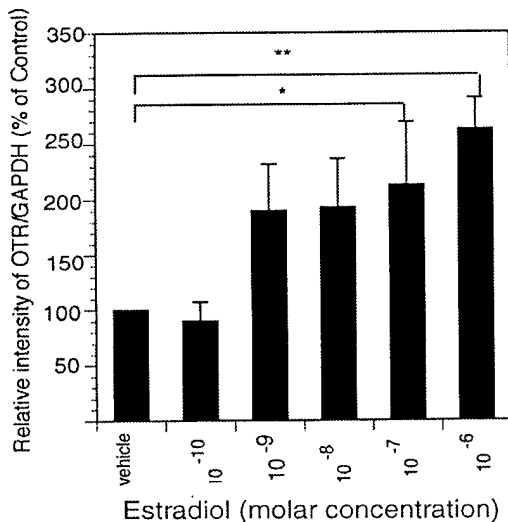


Fig 5. MCF-7 cells were grown to confluence and exposed to OT or vehicle for 24 h. [^3H]-thymidine incorporation was decreased by OT. ** control vs 10^{-6} M and 10^{-7} M $p < .05$ Fisher's PLSD.



The effects of estrogen and progesterone upon OTR were also tested in MCF-7 and HS578T cells. Cells were exposed to vehicle or E_2 (10^{-10} to 10^{-6} M) for 24 h or progesterone and harvested for measurement of the OTR-PCR product. In both cell lines, E_2 increased OTR in a dose response fashion, **fig 6**,

Fig 6. Estradiol increased the abundance of the OTR PCR product in MCF-7 cells. Cells were exposed to estradiol for 24 h. The figure shown represents 3 separate experiments. * control vs 10^{-7} M E_2 , $p < 0.01$, control vs 10^{-6} M, $p < 0.001$, Fisher's PLSD.

whereas P had the opposite effect, **fig 7**. Additional findings suggest that the E-induced increase in the OTR PCR product is accompanied by an increase in OTR binding and the P-induced decrease is associated with a decrease in binding. In collaboration with Dr. Phillip Rauk, University of Pittsburgh, we measured OTR binding by radiolabeled ornithine vasotocin (OVT) competitive binding assay in MCF-7 and HS578T cells, and the results are shown for HS578T cells, **fig 8 and 9**. E₂ treatment of these cells for 24h increased OTR binding by 30-40% compared to vehicle-treated cells, **fig 8**, whereas P decreased binding by 30-40%, **fig 9**. These finding suggests that the E₂- or P-induced alterations in the OTR PCR product translates into functional changes in receptor binding as well.

We also determined if the growth inhibitory effects of OT are mediated by PKC. PKC mediates the effects of OT in uterine cells, in which the signal transduction pathways for the action's of OT have been most extensively studied To determine whether PKC mediates the growth inhibitory effects of OT in human breast cancer cells, we incubated HS578T cells with an activator of PKC, phorbol dibutyrate, PDBU 100 nM, vehicle, or OT 100nM and measured ³[H]-thymidine

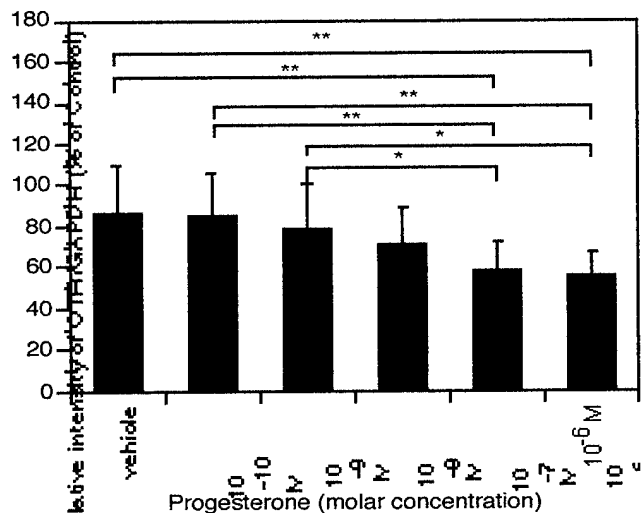


Fig 7. Progesterone treatment of MCF-7 cells. Progesterone decreased the abundance of the OTR PCR product in MCF-7 cells. Cells were exposed to progesterone for 24 h. The figure represents 3 separate experiments. **control and 10⁻¹⁰M vs 10⁻⁷M and 10⁻⁶M and 10⁻¹⁰M p < .005. * 10⁻⁹M vs 10⁻⁷M and 10⁻⁶M p < .05, Fisher's PLSD.

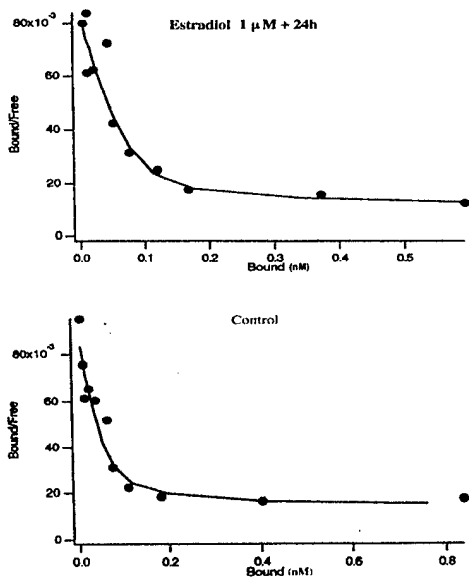


Fig 8. OTR binding in HS578T cells grown in the presence (upper) and absence (lower) of E₂ (10⁻⁶ M for 24 h). For the non-E₂-exposed cells, the Kd = 0.70 nM and Bmax = 331 fM per mg protein were lower than for the E₂-exposed cells, Kd = 1.02 nM and Bmax = 331 fM per mg protein. Scatchard plot measures total binding using IGOR (Statfit 1.1) by Wavemetrics Inc.

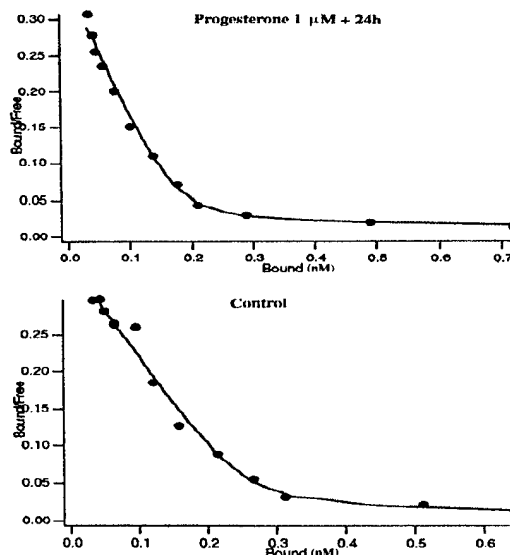


Fig 9. OTR binding in HS578T cells cultured in the presence (upper) and absence (lower) of progesterone (10⁻⁶ M) for 24 h. For the non-progesterone exposed cells, the Kd = 0.74 and Bmax = 149 fM/mg protein were higher than for progesterone-exposed cells, Kd = 0.57 and Bmax = 103 fM/mg protein. Scatchard plot measures total binding using IGOR (Statfit 1.1) by Wavemetrics Inc.

incorporation. Both OT and PDBU decreased ^3H -thymidine incorporation compared to vehicle-treated cells, **fig 10**. We also preincubated cells with an inhibitor of PKC, myristoylated PKC inhibitor, 100 nM and 10nM, prior to OT 100 nM and measured ^3H thymidine incorporation. The growth inhibitory effects of OT were prevented in a dose response fashion.

In summary, OT suppresses the growth of MCF-7 and HS578T cells and decreases the incorporation of ^3H thymidine in MCF-7 and HS578T cells, suggesting that OT may protect against cellular proliferation. OTR is expressed in several human breast cancer cell lines, MCF-7, BT-20 and HS578T as well as in cancerous human breast cells removed from women at the time of mastectomy for breast cancer. Immunohistochemistry localizes OTR to epithelial-derived cells of breast cancer and myoepithelial cells of non-cancerous breast tissue. Estrogen increases OTR expression and receptor binding and progesterone inhibits expression and binding. PKC pathways appear, in part, to mediate the effects of OT in human breast cancer cell lines.

The initial studies were done in human breast cancer cell lines because of the ease of obtaining these cells. Because we can also grow human mammary epithelial cultures (HMECs) organotypically, our next step is to conduct similar studies in HMECs.

HMECs provide us with a powerful tool by which to study the effects of OT upon various growth characteristics of tumor and normal (reduction mammoplasty samples) mammary tissues. In culture, the cells form domes, buds and ducts, thus mimicking the architecture of the mammary gland **fig 11**. The cultures are monitored with real time photography, which provides a dramatic example of the phenotypic differences in growth rate and patterns between normal and neoplastic mammary tissue. The cell types in normal and cancerous cultures have been extensively characterized by transmission and scanning electron microscopy.

We will establish fresh non-diseased human mammary epithelial cell cultures [HMEC] from reduction mammoplasties. These cultures will be derived from a variety of women and will span several age groups to include pre- and post menopausal women, because breast cancer can occur at either stage of life. The samples taken from the breast reduction mammoplasties will be noted for parity status and will also include the time in each subject's cycle when the surgery was performed. Significant differences in the morphology of breast glandular tissues can be manifested at different hormonal states during the monthly cycle. These tissues will be used to study the effects of OT in **normal** mammary cultures.

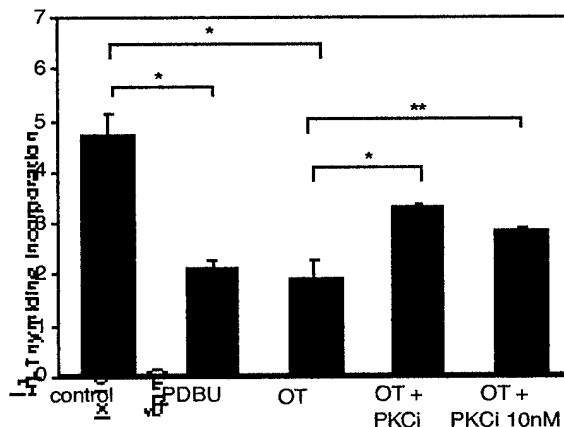


Figure 10. HS578T cells were treated for twenty-four hours with various test agents. ^3H -thymidine incorporation approximates DNA activity. There was a statistically significant decrease of ^3H -thymidine uptake after treatment of the cells with PDBU (phorbol dibutyrate, a protein kinase C activator) and OT (oxytocin). Furthermore, there was a statistically significant inhibition of this effect when cells were pre-treated with PKCi (myristoylated PKC activator). (* = $p < 0.001$; ** = $p < 0.05$, Fisher's PLSD)

Fig 11. Scanning electron micrograph of normal epithelial "dome" in primary culture derived from breast reduction mammoplasty (14 days) at high power. [Reproduced in the Appendix].

monthly cycle. These tissues will be used to study the effects of OT in **normal** mammary cultures.

Unlike most other *in vitro* HMEC systems, Dr. Latimer's novel system of developing organotypic culture does not involve the purification of epithelial cells or glandular structures. We are therefore reasonably certain that the **myoepithelial** cells, which express OTR in **normal** mammary tissue, will be present in the normal primary cultures, a critical requirement for studying OTR in **normal** tissue. PCR of RNA from human breast reduction mammoplasty illustrates a 397 bp OTR PCR product, **fig 12**. Immunocytochemistry of, **fig 13**, using the OTR antiserum illustrates the presence of OTR in **non epithelial** cells of **normal** HMECs.

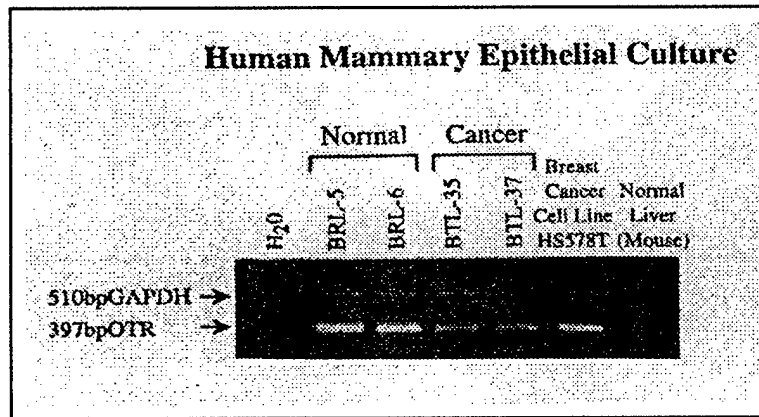


Fig 12. Samples of RNA (1 mcgm) from normal HMECs (BRL-5 and BRL-6, obtained from women undergoing reduction mammoplasty) and cancerous (BTL-35 and BTL-37, obtained from women undergoing mastectomy) were reverse transcribed with OTR and GAPDH specific primers, and each transcript was amplified by PCR. A 397-bp OTR PCR product was identified in the normal and cancerous HMECs. Also shown are lane 1, water; lane 6, HS578T cells; and lane 7, normal liver.

In addition to the normal tissue, breast tumor tissue and tumor adjacent tissue will also be placed into Dr. Latimer's *in vitro* culture system for the establishment of primary cultures. We have identified the 397 OTR PCR product in cancerous HMECs, **fig 12**. Primary breast tumor cultures in this system manifest as wide a variety of doubling times as the tumors themselves. In addition depending on the stage of the tumor, many of these primarily cultured cells are mobile rather than capable of forming stable epithelial-type architecture associated with the normal glandular structures formed by non-diseased breast tissue. Both normal and tumor breast cultures once established are long lived (3-4 months at least) and can be manipulated by changing the hormonal and growth factor milieu. Although these cultures are delicate to set up initially, they are quite stable after the cells have created their own extracellular matrix and the culture medium can be adjusted for experimental purposes.



Fig 13. Normal HMEC stained with an antiserum to OTR at 1:10,000 dilution. Positively-stained cells are identified in the non-epithelial precursors of the ductal structures. 40X magnification. [Reproduced in the Appendix]

Transmission electron microscopy and immunocytochemistry using antisera specific for epithelial cells (such as epithelial membrane antigen, EMA, **fig 14**) have both been used to identify the components of the cultures. HMECs appear to reiterate the developmental ontogeny of ductal and lobular differentiation from small epithelial “mammospheres” to complex branching ducts, **figs 15 & 16**.



Fig 14. Normal HMEC stained with an antiserum to epithelial membrane antigen (EMA). Positive staining identifies the epithelial cells in the culture.

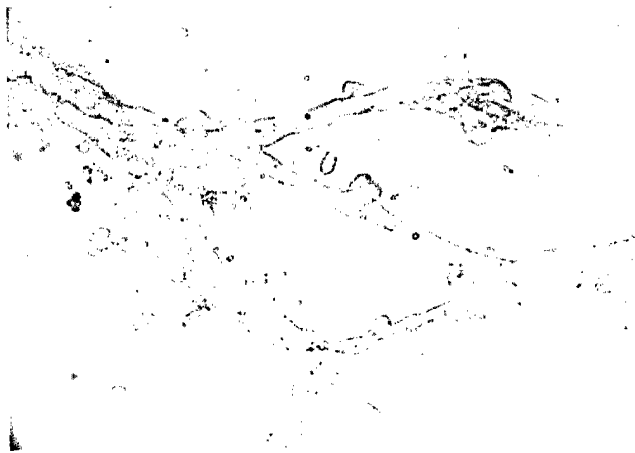


Fig 15. Differential interference microscopy of normal HMEC. Branching ducts are identified after 15 days in culture. 100X magnification.

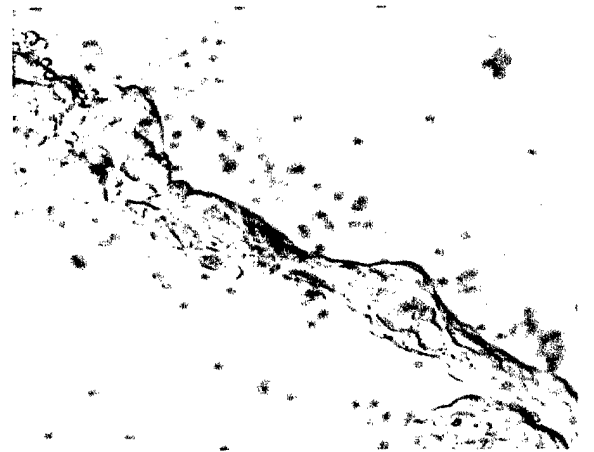


Fig 16 Differential interference microscopy of normal HMEC. High power view of duct formation after 15 days in culture. 200X magnification.

It appears that acquisition of the OTR by the mammary epithelial cell is an accompaniment of malignant transformation, when under normal conditions OTR is expressed in myoepithelial cells. Mammary cancer may represent a de-differentiated state of an original mutated cell. The epithelial cell which becomes malignant (and which is OTR positive) may de-differentiate toward a stem cell which has the capacity to form either a myoepithelial or luminal epithelial cell. The tumors that develop along myoepithelial lines may express OTR. Because there are several steps in the differentiation process, some cells may express OTR whereas others may not. HMECs will provide a novel organotypic system whereby we can study this hypothesis.

***IN VIVO* STUDIES**

Companion studies are also being performed in the OT "knockout" mouse. These OT "knockout" mice, derived from 129 mice with a CB 57/B16 background, have not processed OT and thus the mammary glands are not exposed to OT at any stage of development. The animals are currently being studied to test the hypothesis that diminished OT and the absence of its effects upon the mammary gland, may in part, predispose the breast to neoplastic change and that exposure to OT may be protective against breast cancer.

To date we have sacrificed OT "knockout" mice and normal wild type mice (with no deficiency of OT) at the following months after delivery: 1.5, 3, 6, 9, 12, 18. Over the course of the next 18 months, we plan to sacrifice animals at 24, 30 and 36 months. The histological examination of the mammary glands is presently being performed.

In addition we are breeding OT "knock out" mice with MMTV-infected CH3 mice that are predisposed to the development of mammary cancer and determine the prevalence of mammary neoplasia in the progeny of MMTV-infected mice that are bred with OT "knockout" versus wild type mice. These studies are ongoing and will span the next 18 months.