

Breast Cancer Prevention

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Our laboratory's major interest is the prevention of breast cancer through the induction of differentiation of the breast or mammary gland by optimizing the use of the hormones of pregnancy. A high susceptibility or "high risk" window exists early in life, during a period between the initiation of ovarian function and the first pregnancy. During the "high risk" period the mammary gland parenchyma exhibits specific morphology, cell kinetics and genomic characteristics that continuously vary under the influence of ovarian and pituitary hormones. Sudden and profound changes occur during pregnancy, under the influence of embryonic and placental hormones. Pregnancy and treatment of virgin rats with the placental hormone human chorionic gonadotropin (hCG) inhibit cancer development. This hormone alone induces the differentiation of the mammary gland through a progressive process of activation of genes associated with proliferation, differentiation, and apoptosis. In addition, hCG activates the expression of tumor suppressors, such as p53 and inhibin, and induces acetylation of histones H3 and H4 in the normal mammary gland and in human breast epithelial cells *in vitro*.



The fact that mammary tumors over express DNA methyltransferase led us to postulate that hCG might inhibit mammary carcinogenesis by inducing re-expression of genes silenced by DNA methylation, particularly at CpG islands. Our goal is to utilize this model as a novel strategy for breast cancer prevention. What is required first is elucidating whether hCG inhibits cancer development through the induction of either a specific genomic imprinting or a stable pattern of gene transcription mediated by DNA methylation. We are comparing the efficacy of hCG in activating the tumor suppressor p53 with steroid hormones, a DNA methyltransferase and a histone deacetylase inhibitor. The identification of the optimal hormone or compound(s) for re-expressing genes epigenetically inactivated will provide the basis for defining epigenetic patterns of gene inactivation in tumors and will lead to the optimization of the use of hormones that target epigenetic silencing.

This knowledge will serve as the basis for interpreting the genomic and proteomic profiles of breast epithelial cells and fluids recovered from ductal lavage, fine needle aspirate, or nipple aspirate fluids obtained from women carrying germline mutations in the BRCA1/BRCA2 genes or that had received radiotherapy for Hodgkin's disease, two high-risk groups who have greater probabilities of developing bilateral and hormone-insensitive tumors. The identification of a specific "high breast cancer risk signature" will allow us to evaluate the response of the breast epithelium to the hormonal treatments that we have experimentally proven to reduce breast cancer risk.

Methylation of the estrogen receptor alpha by pregnancy and human chorionic gonadotropin.

I.H. Russo, Liu, in collaboration with Fernandez,[§] J. Russo[§]

Our work is based on epidemiological and clinical observations that breast cancer incidence is markedly reduced in early parous women, and in our findings that this protective effect results from the differentiation of the breast, which is accompanied by reduction in the rate of cell proliferation and downregulation of the estrogen receptor alpha (ER α) in breast epithelial cells. We have induced similar effects in the virgin rat mammary gland by treatment with the placental hormone hCG. The role of estrogen in light of the downregulation of its receptor alpha during pregnancy- or hCG-induced differentiation is unclear. In primary breast cancer, on the other hand, the expression of the ER α gene is absent due to the aberrant methylation at CpG islands.

In order to determine whether downregulation of the ER α occurring during hormonally-induced differentiation of the breast is the result of the addition of a methyl group to a cytosine residue at the CpG islands in the regulatory region of gene, we evaluated the expression of the receptor by RT-PCR and the methylated status of four CpG islands of the rat ER α . RNA and genomic DNA were extracted from the mammary glands of three groups of Sprague Dawley rats: Group I (or hCG) consisted of two subgroups of virgin rats that were daily inoculated with 100IU urinary (u)hCG (Profasi) (Ia) or (Gonacor) (Ib) for 18 days each; Group II (or Pregnancy) consisted of two subgroups of pregnant rats that were euthanized at 18 (IIa) or 21 (IIb) days of pregnancy; and Group III (Control) consisted of age-matched virgin rats that received a daily injection of vehicle for 18 days. The methylation status of ER α in the mammary glands was examined using the sodium bisulfite conversion, amplification by methylation-specific PCR and genomic sequencing. Our results indicated that the density of methylation in CpG islands in 5' exon 1 was significantly higher in the mammary glands of both hCG and Pregnant Groups than in the Control Group, an indication that this site exerts a functional effect on gene expression. The downregulation of the ER α gene expression occurring during differentiation of the mammary gland indicates that the methylation of the estrogen receptor is a differentiation dependent process whose role in the

protection of the breast from cancer initiation requires further study.

Identification of molecular biomarkers of cancer risk in cytologically normal breast epithelial cells.

I.H. Russo, Wang, Balogh, Sheriff, in collaboration with Masny,[§] Daly,[§] Torosian,[§] J. Russo[§]

Women who are carriers of BRCA/BRCA2 mutations have a lifetime risk of breast cancer of 70 to 85%. BRCA1 mutation carriers are more likely to develop tumors that are estrogen receptor negative and less likely to benefit from anti-estrogen chemopreventive treatments, but it is not possible currently to predict which of these women will develop breast cancer. The use of molecular approaches may allow us to identify the "high risk" genomic signature of the cytologically normal breast epithelium of women at high risk for breast cancer. This signature will serve as an intermediate biomarker for evaluating the response of the breast to novel chemopreventive agents. For this purpose we analyzed pure epithelial cell populations obtained by laser capture microdissection (LCM) utilizing cytological smears of the normal human breast epithelial cell line MCF-10F. We captured and isolated RNA from sets of 20, 200, and 2000 cells. Purified RNA was measured by fluorometric quantitation and amplified using PCR. Concentrations of a18S housekeeping gene were compared with those of the breast epithelium specific genes for fat milk globule membrane antigen and whey acidic protein. Our results led us to conclude that RT-PCR is sensitive for detecting RNA from small numbers of LCM selected cells. These observations confirmed the usefulness of the application of LCM to cytospin preparations for obtaining pure cell populations for RNA extraction and of PCR RNA amplification for cDNA microarray analysis and of RT-PCR for gene expression level quantification. These studies will allow us to perform genomic hierarchical cluster analysis and bioinformatics for patient risk assessment.

Human chorionic gonadotropin (hCG) induces specific molecular pathway of cell differentiation in the mammary gland of *Macaca fascicularis*.

I.H. Russo, Mailo, Balogh, Sheriff, in collaboration with P.A. Russo,[§] Appt,^a Blair,^a Cline,^a Clarkson,^a J. Russo[§]

Epidemiological, clinical, and experimental

evidence indicate that the risk of developing breast cancer is markedly reduced in early parous women and in rodents treated with hCG. The protective effect of hCG causes differentiation in the breast and imprints in the mammary parenchyma of rodents a specific genomic signature associated with lifetime resistance to neoplastic transformation. The cynomolgus macaque (*Macaca fascicularis*), whose reproductive physiology faithfully replicates the human condition, was selected for evaluating the effect of hCG treatment on mammary gland genomic expression. Seven sexually mature cycling *Macaca fascicularis* were administered intramuscularly 200IU hCG/kg of body weight three times a week during 3 months. Mammary gland biopsies were performed at four different time points: 1) beginning of treatment; 2) end of treatment; 3) three months post-treatment; and 4) six months post-treatment. Mammary tissues were processed for histopathology and RNA extraction. The RNA of each animal obtained at each time point was individually extracted, amplified, and hybridized to human cDNA microarrays containing 40,000 features by triplicate, using a universal human RNA as reference. Gene expression analysis revealed that the mammary RNA of all the *Macaca fascicularis* hybridized to 73.5% of the human genes present in the array. Three animals with satisfactory glandular material at all times are reported here. Gene expression varied as a function of time of treatment in two patterns: Pattern A, genes expressed pre-treatment, overexpressed at 3 months post-treatment, and downregulated 6 months post-treatment, and Pattern B, genes downregulated pre-treatment, present or upregulated at the end of treatment, and significantly overexpressed at 3 and 6 months post-treatment. Pattern A included *Ephrin B3*, *BCL-2* associated X protein, and *Homeobox C9* genes. Pattern B included genes involved differentiation, i.e., *CYLC1*, *BLNK*, *DAZAP1*, *NDRG2*, *FLT1*, *PRM1*, *SMURF1*, *PPARG*, *PAX8* and *TNESF11*, and in organogenesis, such as *FABP7*, *TCF12*, *TLE3*, *APOE*, *SAS10*, *TNNI3*, *BMPR2*, *TNFSF11*, *UBE3A*, *CUGBP2*, *KRT5*, *EGR2*, *TPM2*, *PTHR1* and *MEF2C*. These studies confirm the usefulness of the *Macaca fascicularis* for the analysis of gene expression and the responsiveness of the mammary gland to hormonal treatments. This species responds, like rodents, to the hCG

inducing activation of genes related to gland development and differentiation.

The expression of clock genes in the rat mammary is regulated by the circadian rhythm.

I.H. Russo, Wang, in collaboration with Moral,[§] J. Russo[§]

The development of the mammary gland is under the control of the hypothalamic-pituitary-gonadal (HPG) axis. Hormones secreted by the ovary and the pituitary and the timing of cell division are in turn affected by circadian rhythms controlled by the master circadian clock located in the suprachiasmatic nucleus (SCN) of the hypothalamus whose 24-hour rhythm is entrained by light reaching the retina. The SCN controls both central and peripheral clock genes as well as the release of gonadotropin and luteinizing hormone releasing hormone (GnRH/LHRH) neurons. Because the stage of development and rate of cell proliferation in the mammary gland are essential for cancer initiation, we postulate that the susceptibility of this organ to be transformed by chemical carcinogens might be, in part, regulated by circadian rhythms, and that disruption in circadian gene expression could result in deregulation of these processes and contribute to tumor development.

This study was designed to characterize the pattern of gene expression in the rat mammary gland during a 24-hour circadian cycle under standard light conditions. Fifty-day-old female virgin Sprague-Dawley rats were maintained in a light controlled animal facility with a 12-hour light/12-hour darkness cycle. After 2 weeks in this environment three animals were euthanized for the collection of mammary tissues at six time points during a 24-hour period. Total RNA was isolated from frozen mammary glands and quantified by Nanodrop. RNA integrity was assessed by capillary electrophoresis RNA chip. Gene expression by real time RT-PCR was tested for the clock genes: *Per1*, 2 and 3, *cry1* and 2, *bhlhb2*, *Sharp 1*, *bhlhb3*, and *Sharp2*, and *clock*. Maximal level of expression was observed at 8:00 p.m. in *bhlhb3*, *Per3*, *Per1*, *cry2*, *clock*, and *Per2* and four hours later in *bhlhb2* and *cry1*. All clock genes tested exhibited the minimal level of expression at between 8 and 12 hours. Our results demonstrate for the first time that the rat mammary gland expresses clock genes that cycle in response to circadian

rhythms. This approach will open new avenues for understanding the modulation of the response of the mammary gland to hormones under environmental influences such as light, which might act as endocrine modulators.

Effect of circadian disruption on the expression of clock genes in the suprachiasmatic nucleus (SCN), mammary gland and liver.

I.H. Russo, Wang, in collaboration with Rea,^b Figueiro,^b Bullough,^b Possidente,^c Moral,[§] Vanegas,[§] Fernbaugh,[§] J. Russo[§]

The circadian system is formed by a network of structures that control the body's biochemistry, physiology, and behavior in a daily rhythmic oscillation based on 24-hour intervals. For studying this system, which is under the control of the hypothalamic suprachiasmatic nucleus (SCN) and synchronized by light reaching the retina, we utilized thirty virgin Sprague Dawley rats that were maintained under a light-dark cycle of 12 h light :12 h dark (L:D) to determine whether the mammary gland expresses

peripheral clock genes and if so, whether they express circadian rhythmicity under the control of the SCN master circadian clock. Our results are the first to demonstrate that clock genes in the mammary gland exhibit a circadian rhythm. Additional female Sprague-Dawley rats were randomized at the age of six weeks to study disruptions of the circadian rhythm by altering times of light exposure. Disruption of the light cycle revealed that the expression of clock genes remained shifted about 8 to 12 hours and highly expressed at daytime instead of at nighttime after two days of having returned to the normal LD cycle. Photoperiod disruption reduced clock gene synchrony between liver and mammary tissues and strengthened the internal mammary clock gene synchrony. Since clock gene expression is emerging as an important factor in cell cycle regulation and development, we postulate that light-induced circadian disruption of clock gene regulation could affect cell proliferation and mammary cancer initiation.

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