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14. ABSTRACT Background and Hypothesis: Pregnancy associated breast cancer (PABC) has higher metastatic potential. We propose that the process of mammary gland involution following pregnancy co-opts programs of wound healing and this pro-inflammatory milieu promotes metastasis. The following aims are proposed. Aim 1. Validate in human breast tissue, that breast involution has a pro-inflammatory component. Results: CD45 and CD68 positive cells are increased in human involuting breasts, consistent with a pro-inflammatory program. Aim 2. Investigate the hypothesis that PABC in women will be characterized by expression of negative prognostic stromal markers (desmoplasia) and correlate with clinical data and outcomes. Results: work to begin year 2. Aim 3. Using animal models for human PABC, determine whether mammary tumors that develop in the context of involution have increased desmoplasia and metastases. Results: Four new models for PABC are in development with promising preliminary results. Impact on Breast Cancer Research and Patients: The identification of mammary gland involution as the mediator of PABC metastasis identifies a new window for targeted therapies directed at decreasing the pro-inflammatory milieu of the involuting breast.					
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Introduction

Pregnancy associated breast cancer (PABC), defined for our studies as a breast cancer diagnosed within two years after a completed pregnancy, but not during pregnancy, portends a higher metastatic potential, and therefore a poorer prognosis. We propose that the process of mammary gland involution following pregnancy that returns the gland to its pre-pregnant state, co-opts programs of wound healing. The pro-inflammatory milieu that results, while physiologically normal, promotes metastasis. In support of our hypothesis, we have found that the microenvironment of the involuting rat mammary gland shares similarities with wound healing. Further, ECM isolated from rat mammary glands undergoing involution induced tumor cell invasion in vitro and metastasis in a xenograft model, whereas ECM isolated from glands of quiescent nulliparous rats did not. To address our hypothesis, we propose to validate in human breast tissue, that breast involution has a pro-inflammatory component, using immunohistochemical approaches. We will investigate the hypothesis that PABC in women will be characterized by expression of negative prognostic stromal markers (desmoplasia) and correlate with clinical data and outcomes. Because animal models for PABC do not exist, we propose to validate whether two animal models will be suitable for the study of PABC; a xenograft model for human PABC using MDA-MB-231 cell and the MMT bi-transgenic murine model of spontaneous mammary carcinoma. We predict that tumors in the involution microenvironment will be desmoplastic and will readily metastasize compared to tumor cells in the nulliparous environment.

Body:

Task 1 Obtain paraffin-embedded breast tissue from cases of women under the age of 45 for analysis of non-cancerous, involuting v. non-involuting tissue and for analysis of PABC v. non-PABC cases. (Months 1-12)

Task 1a) Regulatory issues – Approval from the Colorado Multiple Institutional Review Board (protocol 05-0958) was obtained on 5/15/2006 to collect the required samples from the University of Colorado Breast Center and Denver Health Medical Oncology Breast Center.

Results to date: Task completed. However, the DOD review and approval of our Human Protocol were not completed until April 17, 2008, thus start of tasks requiring human tissue were delayed until this final approval was received.

Task 1b) Identification of relevant cases and their histological specimens. **Results to date:** Task Ongoing. Postdoctoral fellow Traci Lyons has completed all COMIRB training. To identify PABC and control cases, using the University of Colorado database, Dr Lyons is actively reviewing all medical records for breast cancer patients diagnosed at age 45 years or younger.

Task 1c) Norwegian Data Set - submission of a full protocol and clearance of Norwegian regulatory bodies will be performed in the first 6 months of the project. **Results to date:** The subcontract between the Regents of the University of Colorado and the University of Bergen was formalized on 5-23-08. The official start date of the subcontract is 6-15-08.

Task 1d) Norwegian pathologic specimens – once relevant cases of PABC have been identified from the existing data set, the tissue blocks will be requested and slides prepared and shipped to the Schedin lab for analysis. **Results to date:** Inclusion criteria for PABC and control cases have been defined (see Table 1) and we anticipate receiving tissue sections from Dr. Albrektsen beginning August, 2008.

Task 1e) We have already obtained 10 cases of involuting human breast tissue/PABC from Dr. Man, Chief Breast Pathologist, Air Force Institute of Pathology, as noted in the preliminary data results. **Results to date:** these tissues were used to obtain preliminary data and will not be further evaluated as part of this DOD funded study.

Table 1	PABC < 40year unipara	NON-PABC < 40 year	
Total number	54	212	500
Year of diagnosis			
< 1993 (55-92)	43 (79.6)	164 (77.4)	365 (73.0)
≥ 1993 (93-99)	11 (20.4)	48 (22.6)	135 (27.0)
Age at diagnosis			
20-24 yr	6 (11.1)	0 (0.0)	17 (3.4)
25-29 yr	18 (33.3)	1 (0.5)	52 (10.4)
30-34 yr	16 (29.6)	48 (22.6)	155 (31.0)
35-39 yr	14 (25.9)	163 (76.9)	276 (55.2)
Clinical stage			
I	26 (48.1)	116 (54.7)	265 (53.0)
II	24 (44.4)	82 (38.7)	176 (35.2)
III	3 (5.6)	2 (0.9)	21 (4.2)
IV	1 (1.9)	12 (5.7)	38 (7.6)
Maternal age at 1 st birth			
< 20 yr	0 (0.0)	53 (25.0)	
20-24 yr	8 (14.8)	104 (49.0)	
25-29 yr	20 (37.0)	55 (25.9)	
≥ 30 yr	26 (48.1)	0 (0.0)	
Year of birth (cohort)			
1925-39	7 (13.0)	58 (27.4)	136 (27.2)
1940-49	12 (22.2)	65 (30.7)	119 (23.8)
1950-59	16 (29.6)	77 (36.3)	150 (30.0)
1960-69	19 (35.2)	12 (5.7)	89 (17.8)
1970-79	0 (0.0)	0 (0.0)	6 (1.2)

Table 1 (per March 12th, 2008)
Number of potential participants within predefined exposure categories

PABC-cases (young uniparous women):

- Uniparous women, age at diagnosis < 40 years, diagnosed within 2 years after date of birth of child

Non-PABC cases (young, nulliparous and uniparous women):

- nulliparous women, age at diagnosis < 40 years
 - uniparous women, age at diagnosis < 40 years, diagnosed ≥ 10 yrs after birth

Task 2 – Obtain linked clinical data from the histopathologic samples from Task 1 (Months 1-18).

Task 2 a) Clinical abstraction of the electronic and paper chart data for local cases has begun and will continue through the first year and a half of the project, reflecting the length of time required to fully obtain the needed clinical data for linkage and outcomes analysis with the immunohistochemical pathologic results. **Results to date:** Task ongoing.

Task 2b) For the Norwegian cases, after appropriate regulation ensuring human subjects protection is obtained, cases will be abstracted from their existing clinical data set on PABC and de-identified clinical data sent to Drs. Schedin and Borges for verification of inclusion of individual cases into the data set. **Results to date:** Cases have been abstracted and inclusion criteria defined (see Table 1).

Task 2c) De-identified clinical linked data to the 10 cases provided from the Air Force Academy will be obtained. **Results to date:** As mentioned in Task 1e, these cases are not part of our ongoing study and will not be referred to in subsequent reports.

Task 2d) The local and Norwegian cases will be merged in to a single de-identified data set for further use. **Results to date:** Upon receipt of the Norwegian cases, we will merge the local and Norwegian cases into a single file. To date, no work has been accomplished on this task.

Task 3 – Histological evaluation of non-cancerous, involuting v. non-involuting human breast tissue samples, (Months 3-18).

Task 3a) Cases from women under the age of 45 who have had a pregnancy within the last two years and can be verified to have been recently lactating, either by clinical history or the clear presence of lactational breast change by histology will be identified, as will non-parous, non-lactating controls. Twenty cases of lactating and non-lactating specimens will be identified.

Results to date: Table 2 summarizes the number of patient age-matched breast biopsies obtained in each of the distinct reproductive states; nulliparous, pregnant, lactating, involuting and fully regressed (parous).

<u>Source</u>	<u>Nullip</u>	<u>Pregnant</u>	<u>Lactation</u>	<u>Involution</u>	<u>Regressed</u>
Paraffin Embedded (UC)	0	10	15	11	6
Paraffin Embedded (UC Shine Study)	8	0	0	0	9
Total	8	10	15	11	15

Table 2

Inventory of normal, non-cancerous tissue obtained from human breast biopsies from two UC sources representing never-been-pregnant (nullip), pregnant, lactation, actively involuting and fully regressed alveoli.

Task 3b) Paraffin-embedded slides will be coded so that subsequent analyses will be performed in an unbiased manner.

Non-cancerous, adjacent normal breast tissue will be stained with the following: common leukocyte antigen CD45, macrophage specific marker CD68, endothelial cell marker von Willebrand factor, reactive fibroblasts marker smooth muscle actin, and for MMPs 2, 3 & 9, COX-2, and oncofetal ECM protein tenascin-C and fibrillar collagen. **Results to date:** Our focus in year one has been to collect fixed human breast tissue from women who were pregnant, lactating or weaning. We have stained these samples for CD45 and CD68, and have used breast cancer cases as positive controls, with results described below.

Task 3c) Complete images from these sections will be digitally captured at high microscopic magnification (400X) using a tiling image system (Syncroscan) and evaluated by a pathologist using a personal computer with 4 GB RAM memory, IntelPentium 4 GHz and AdobePhotoshop. We will use a 1mm grid pattern overlapped on the histological images by using layers in PSD (portable document format) files. A color coding system integrated to a customized computer program will create a database integrating data from lesions and topography from each grid. This will allow a correlation between the intensity and topographic distribution of chronic inflammation with involuting and non-involuting breast tissue. **Results to date:** An alternative quantitative histological approach was undertaken to evaluate normal human breast tissue because we wanted to focus on individual lobules in the breast. Specifically, image acquisition was performed using an Aperio Scan Scope T2 System, Vista, CA. Specimens were scanned at a resolution of 1 pixel / 0.5 μ and then down-sampled to a resolution of 1 pixel / 2.4 μ . Images were down-sampled to facilitate the analysis of entire specimens. Image analysis operations were primarily performed through NIH ImageJ; NIH, Bethesda, MD. Using the ImageJ library, custom written plug-ins were written in the Java programming language. Image analysis algorithms were designed to identify and quantify the different immunohistochemical characteristics of the specimens. Specific areas (alveolar lobules) were removed from the larger tissue specimen using a computer assisted 'lasso' tool and placed into a new file for analysis. Identification of positive signal within the area of interest was accomplished with a colorimetric threshold gating step of the immunohistochemical stain. Image pre-processing procedures are necessary for preparing the image for subsequent analysis steps. Within pre-processing, images are examined for obvious scanning errors and non-specific immunohistochemical staining. If any irrecoverable errors in the tissue processing are found then the specimen is rejected. Simple tissue irregularities or areas of disinterest were manually eliminated from the specimen. Image pre-processing steps also utilize image analysis algorithms for identification of the white field area surrounding a tissue specimen. The contiguous white field area surrounding the tissue specimen is identified through a colorimetric threshold range and then assigned a new predetermined single color value. Once images had been optimized through various pre-processing steps, the images were analyzed using a series of algorithms. Primary Image algorithms rely on identifying immunohistochemical features through colorimetric thresholds. Secondary pass algorithms employ the use of various morphometric quantifications on individual objects. Algorithmic-targeted image analysis provides several benefits over the typically subjective nature of the human eye. Automated algorithmic-targeted image analysis can eliminate intra-observer and inter-observer variability resulting in a repeatable mathematically quantifiable set of data.

Using this approach, we have begun the quantification of CD45 IHC, as a measure of leucocyte infiltration (**Figure 1**) and CD68, as a measure of macrophage content (**Figure 2**) in pregnant, lactating, involuting and fully regressed breast tissue. Preliminary data demonstrates that CD45 and CD68 are elevated during mammary gland involution consistent with our hypothesis that gland regression has a pro-inflammatory signature.

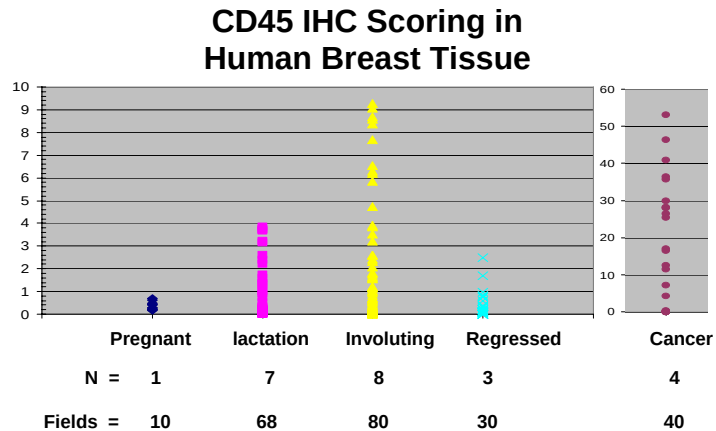


Figure 1. Formalin fixed, paraffin embedded 5µm sections of human breast tissue were stained for CD45, the common leucocyte antigen, by IHC. Using a computer assisted quantification program, the percent positive cells were evaluated in ~10 fields per case.

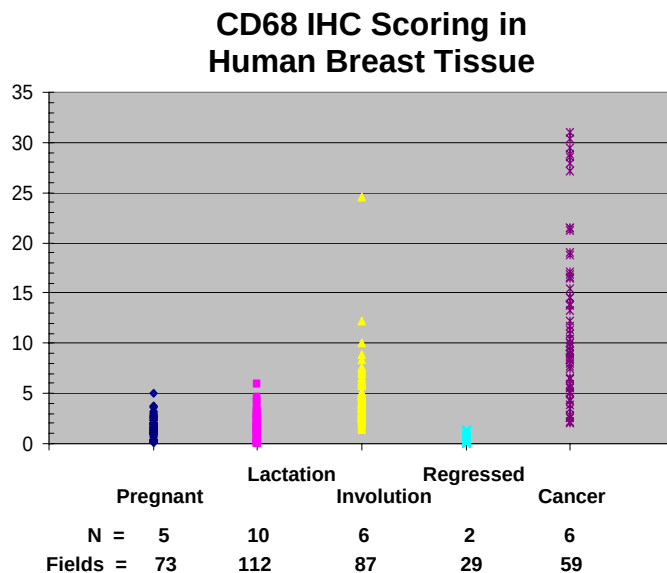


Figure 2. Formalin fixed, paraffin embedded 5µm sections of human breast tissue were stained for CD68, a lysosomal marker in macrophages by IHC. Using a computer assisted quantification program, the percent positive cells were evaluated in ~10 fields per case.

Task 4 – Histological Evaluation of PABC v. non-PABC cases, Months 3-18, and analysis of IHC data and comparison to clinical data set with outcomes analysis, Month 12-16 for involution work, Months 18-24 for PABC work. Results to date: due to the delay in finalizing the Human Subjects protocol and the subcontract with Norway (which have both been finalized) these tasks have been delayed. As a result of these delays, we would like to request a one year no cost extension to the grant. We will have funds to continue this work into year 3 because we have not spent the monies allocated year 1 to this part of the project.

Task 5 – PABC Animal Model Development with xenograft model

Months 6-18

Task 5a) An IACUC approved animal protocol is already in place that covers the research outlined in this task. All animal studies are performed in full IACCUC compliance.

Task 5b-d) Forty, 8-week old female homozygous Rag-1 mice were randomized into two groups with 20 mice per group; nulliparous and involuting. For the involution group, mice were bred. At birth, pup number was normalized to 8 and after 7 days lactation, pups were removed to initiate involution. Two days post weaning, all mice were anesthetized and 2×10^6 MDA-MB-231 cells injected into the fat pad of mammary gland #4. Tumor growth was measured twice weekly. Forty five days post weaning, mice were euthanized, tumors excised and final tumor weight and volume calculated. **Results to date:** In a pilot study, using 10 mice per group, MDA-MB-231 cells injected into an involuting microenvironment produced larger tumors than when the cells were injected into quiescent, nulliparous glands of virgin mice (**Figure 3**).

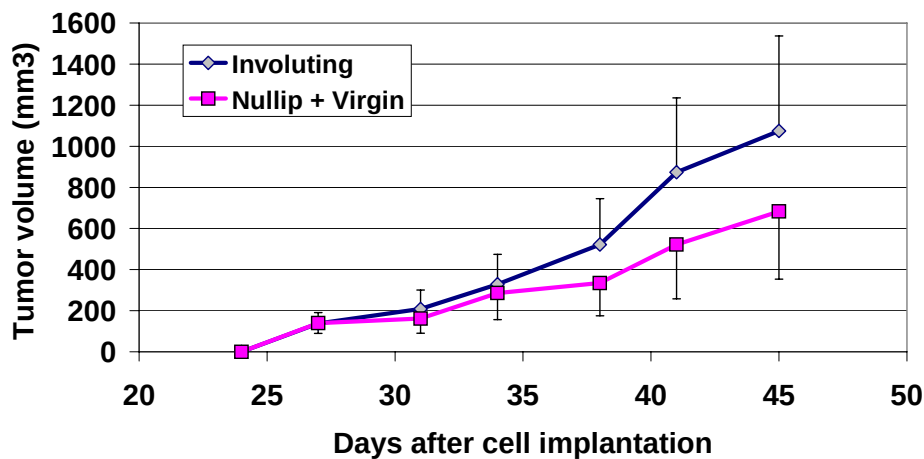


Figure 3. Involution mammary microenvironment promotes tumor growth in vivo. Actively involuting SCID mouse mammary glands were injected with 2×10^6 MDA-MB-231 cells on day two involution. Tumor growth was monitored for 45 days and compared to age matched, nulliparous SCID mice injected with the same number of cells on the same day.

In another study, *MCF10-DCIS* mammary epithelial cells were injected directly into the mammary glands of SCID mice whose mammary glands were quiescent (nulliparous) or actively involuting (N=12 per group). These cells are a derivative of the MCF10A normal mammary epithelial cell line, and have the capability to form human DCIS-like lesions in the mouse [1]. For the involution group, mice were bred; litters normalized to 8 pups and allowed to lactate for 10 days. The pups were then removed to induce involution and 2×10^5 *MCF10-DCIS* cells were injected into the mammary gland on day two of involution. Tumor formation was monitored for 8 weeks, with time-points taken at 3, 4, 6, and 8 weeks. The overall tumor incidence in each group is shown on the graph below (**Figure 4 A**). Tumor number, from each group, over the course of the study is also graphed below (**Figure 4B**).

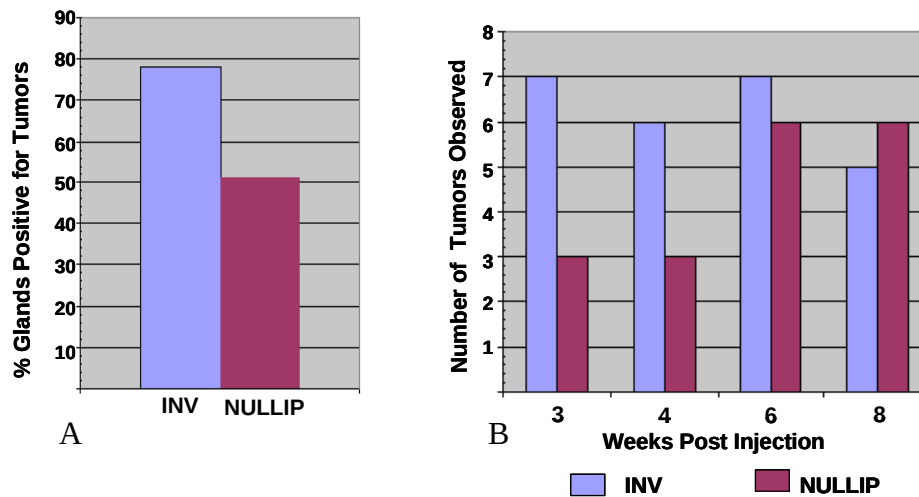


Figure 4. Involution mammary microenvironment promotes tumor growth in vivo. Actively involuting SCID mouse mammary glands (right and left #4) were injected with 2×10^5 MCF10-DCIS cells on day two of involution. Tumor incidence was monitored for 8 weeks and compared to age matched, nulliparous SCID mice injected with the same number of cells at the same time. 8 glands were analyzed per time-point. **A)** Cumulative tumor incidence at study end. **B)** Number of tumors observed in each group over eight week time course

These results indicate that the involution group has an increased tumor incidence over the nulliparous group in this study. The resulting glands from each time-point have been analyzed by H&E for histology, fluorescence in situ hybridization (FISH) to determine species of origin of each type of cell, and for smooth muscle actin (SMA) to confirm DCIS-like lesions. The results of this analysis for one animal are shown below (**Figure 5**).

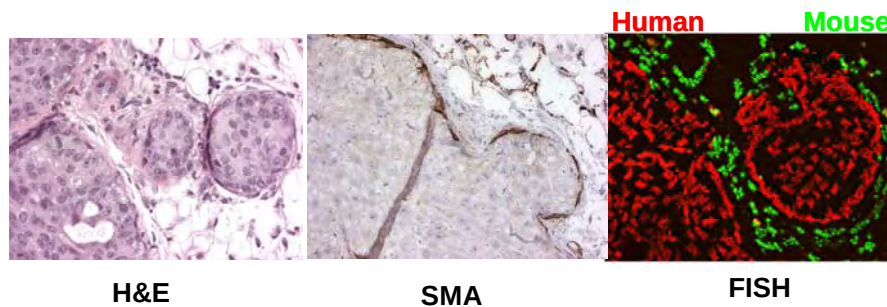


Figure 5. Characterization of tumors formed in MCF10-DCIS mouse xenograft study. Mammary glands were excised from SCID mice injected with human MCF10-DCIS cells and serial sections analyzed for tumor formation by H&E stain for histology, IHC for smooth muscle actin (SMA) with myoepithelial cell layer staining brown, and FISH for species specific COT-1 DNA (red = human and green = mouse).

Overall, this analysis has revealed that not only does tumor incidence appear to be increased in the involution group, but tumor size as well. Preliminary data indicates that the involuting microenvironment supports tumor cell dissemination as well, as a more detailed analysis of the FISH data, on involution samples, revealed human tumor cells migrating through mouse stoma (Figure 6).

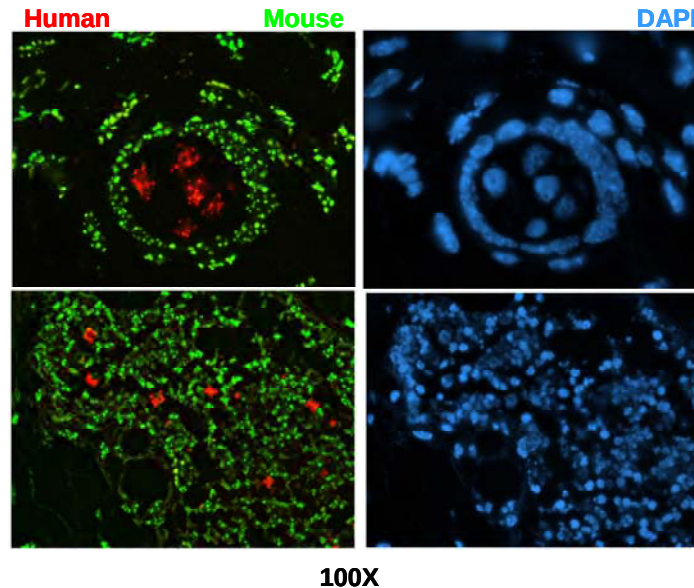


Figure 6. FISH using probes for mouse Cot-1 (green) and human Cot-1 (red) reveals single migratory human tumor cells in mouse 'involution' stroma at sites distant from the primary tumor. DAPI is used to identify all cell nuclei.

Task 5e) At time of euthanasia, liver, lung, kidney and brain tissues are isolated for determination of metastatic lesions. Organ RNA is isolated and human RNA detected by quantitative RT-PCR using human specific primers, using SDS software and analyzed by the CT method. **Results to date:** Secondary site tissue has been harvested. RNA isolation and PCR analyses to be undertaken in year 2.

Task 5f) Characterization of tumor desmoplasia by IHC. The same panel of markers for desmoplastic stroma and angiogenesis described in Task 2 will be evaluated here by the Computer-Assisted Image Analysis method. **Results to date:** This analysis will be undertaken in year 2.

Task 5g) Animal and human PABC IHC data sets will be compared and contrasted. Correlations will be evaluated for statistical significance by Greth Albrektsen. **Results to date:** This task cannot be accomplished until IHC studies are completed.

Task 6 – PABC Animal Model Development with MMT murine Model**Months 6-18**

Task 6a) Submission of an addendum to existing IACUC animal protocol. **Results to date:** IACUC submission completed and approval obtained.

Tasks 6b-d) MUC1 transgenic female mice will be cross bred to male MT.ag bearing mice to result in 1 out of 8 female pups with the double transgenic (MMT). Pups are genetically analyzed for transgene presence using RT-PCR and only female MMT mice are included as experimental animals. This is a rolling breeding scheme, so experimental animals will be used in a sequential fashion as they are available from the breedings. Female MMT will be bred to WT B6 males beginning at 45 days of age of the female mouse. At birth, pup number is normalized to 8 and after 7 days lactation, pups are removed to initiate involution. Foster pups may need to be utilized. **Progress to date:** In the first year, using the breeding scheme described above, we have obtained control (non-PABC) and pregnant MT mice [harboring the polyoma middle t oncogene expressed in the mammary epithelial cells (MEC)] and MMT bi-transgenic (harbor both the polyoma middle t oncogene and the Muc-1 transgene in the MEC). The number of mice in each of these categories is shown below in **Table 3**.

Table 3	Control	Pregnancy 1x	Pregnancy 2x
MT	n=14	n=9	n=8
MMT	n=26	n=5	n=1

Task 6e) Seven days after parturition, mammary gland involution will be induced by pup-weaning. Mammary tumor growth will be recorded as initially defined as any palpable mass arising from the mammary folds. Once tumors are large enough, bi-dimensional measurements from all glands are recorded with calipers. **Results to date:** Our first question was whether mammary tumors, driven by oncogene expression off of the hormone responsive MMTV promoter, would be larger in animals that had undergone a pregnancy. In other words, a potential confounder of this model is that the hormones of pregnancy would upregulate the MMTV promoter, generating data that could appear to be due to pregnancy induction of cancer (our hypothesis) when in fact, the cancer-promotional activity is due to differential MMTV induction between the non-pregnant and pregnant groups. Tumor volumes in the MT model were larger with one pregnancy, but not with two pregnancies, suggesting high inter-animal tumor volume variation rather than a pregnancy effect on tumor volume. In the MMT model, tumor volume was not different between the control and pregnant animals (**Figure 7**).

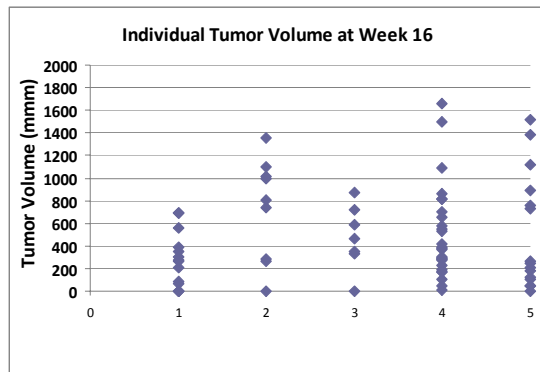


Figure 7. Tumor volumes at week 16 in
 1) control (non-pregnant) MT mice,
 2) MT with one pregnancy,
 3) MT mice with two pregnancies,
 4) MMT control mice and
 5) MMT mice with one pregnancy.

We have also evaluated this model for differences in tumor latency with pregnancy. In the MT model, palpable tumors occur on average two weeks earlier in the pregnant mice compared to non-pregnant controls (**Figure 8**). In the MMT model, differences in latency are not observed between groups. Early time points in both of these models are confounded by the density of mammary tissue in the pregnant animals compared to controls, which may artificially appear to reduce latency.

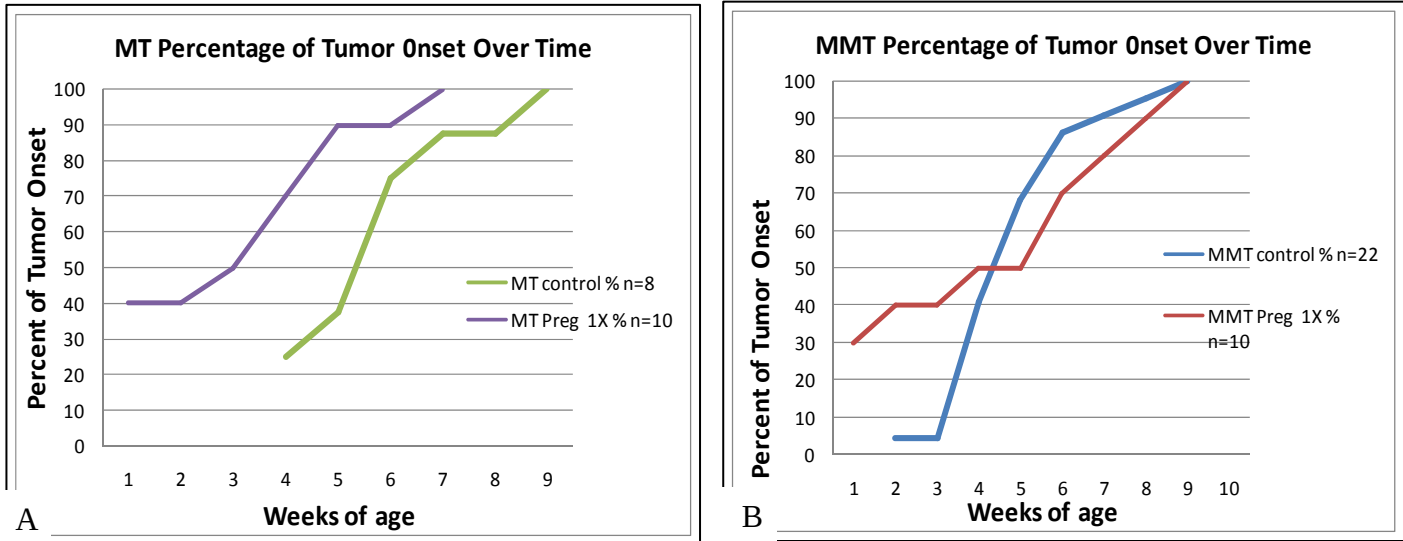


Figure 8. Tumor latency as percent of mice with palpable tumors with time. A) Tumor latency in MT mice with a pregnancy (n=10) is shorter than in control mice (n=8). B) Tumor latency in MMT mice is not different between groups, n=22 for controls and n=10 for mice with pregnancy.

If these preliminary data are confirmed in subsequent studies, i.e. that these models do differ in effect of pregnancy on tumor latency, then one interpretation of these data is that in the MMT model, tumors become non-responsive to the tumor promotional effects of pregnancy because of the transgenic Muc-1 expression. This would suggest that Muc-1 may be expressed at high levels during pregnancy or involution, and it is the high Muc-1 levels that contribute, in part, to the promotional effects of pregnancy on mammary tumors. To address the question of whether Muc-1 is differentially expressed across the pregnancy/lactation/involution cycle, we evaluated Muc-1RNA expression in a mouse microarray data set obtained from earlier studies in the Neville lab. Muc-1 RNA levels are dramatically upregulated over 100 fold with pregnancy and lactation (**Figure 9**). Cumulatively, these data identify Muc-1 as a potential mediator of tumor promotion that occurs with pregnancy in the MT model.

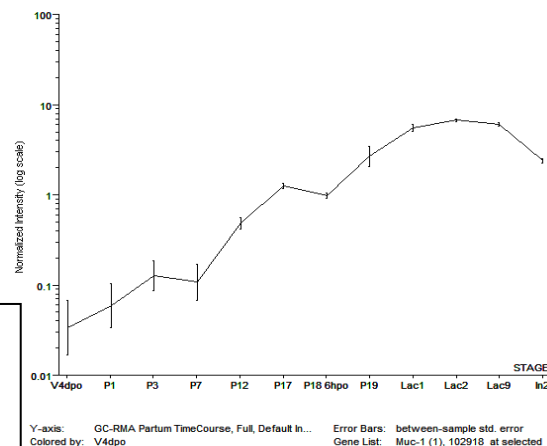


Figure 9. Normalized gene expression pattern for Muc-1 in virgin, pregnant, lactating, and early stage involution mouse mammary glands.

Task 6f) Four weeks post weaning, or as dictated by tumor growth and animal comfort, mice will be euthanized, tumors excised and final tumor weight and volume calculated. Adenomas are confirmed by histological evaluation of all tumors. Final tumor weights (of confirmed adenomas) for the groups are shown in **Figure 10**. While there was a trend for larger tumors in the MT mice with two pregnancies, differences between groups did not reach statistical significance.

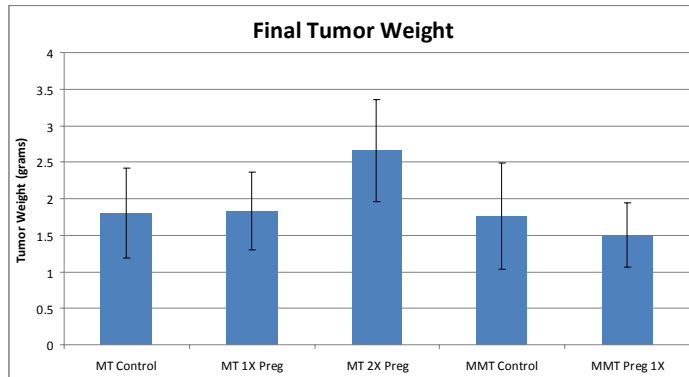


Figure 10. Tumor weight was determined at time of sacrifice. MT control (n=14), MT1Xpreg (N=9), MT2Xpreg (n=8), MMT control (n=26), MMT1Xpreg, n=5. Differences between groups at study end were not observed.

Task 6g) Characterization of tumor desmoplasia by IHC. The same panel of markers for desmoplastic stroma and angiogenesis described in Task 2 will be evaluated here by the Computer-Assisted Image Analysis method. **Progress to date:** These analyses will be undertaken in year 2.

Task 6h) In the MMT bi-transgenic murine model, distant metastases will be detected by quantifying expression of the MTag transgene in RNA isolated from each organ, using methodology described in Task 5. **Progress to date:** Lung metastases have been evaluated by detection of human RNA in 3 MMT control mice, 3 MT mice that have had 2 pregnancies and in one MMT mouse with 1 pregnancy. Even though 2/3 of the control mice had large primary tumors, there was no evidence for lung metastasis in this assay (Table 4). In contrast, in 4/4 mice with two pregnancies, there was evidence of metastases (data highlighted in yellow). Whether this trend is replicated in the mice with a single pregnancy remains to be determined.

ranking by tumor size		tumor size (mmm)	Age at death (weeks)	Average CT values	negative control CT value minus experimental CT value
338613 E1	MMT 1preg	0.417	21.7	27.1	0.76
338625 E4	MT 2 PREG	0.424	20.6	20.8	7.02
338621 E20	MMT Control	0.692	22.6	28.8	-0.98
350413 E2	MT 2preg	0.708	20.4	20.2	7.64
350417 E2	MT 2preg	1.038	20.1	23.6	4.23
328687 E5	MMT Control	1.05	20.7	27.2	0.67
338617 E1	MMT control	1.654	22.6	28.8	-0.95
350413 E3	MMT 2 preg	1.616	19.5	20.4	7.41

Task 6i) Data will be tabulated and compared with nulliparous MMT females for preparation of data presentation and manuscript submission. **Progress to date:** These analyses will be undertaken in year 2.

Task 6j) Animal and human PABC IHC data sets will be compared and contrasted. Correlations will be evaluated for statistical significance by Greth Albrektsen. **Progress to date:** These analyses will be undertaken in year 2.

KEY RESEARCH ACCOMPLISHMENTS: Bulleted list of key research accomplishments emanating from this research.

1. All regulatory documents required for the transfer of human tissues from University of Bergen, Norway to UCD are in place. Inclusion criteria for PABC and control cases have been defined in the Norwegian cohort and location of the corresponding tissue blocks identified (see Table 1). Tissue collection from Norway is ongoing as of 6/15/08.
2. Acquisition of human breast tissue from age-matched nulliparous, pregnant, lactating, involuting and fully regressed breasts.
3. Characterization of human mammary gland involution with demonstration that leucocytes (CD45 positive cells) transiently infiltrate the gland during involution.
4. Demonstration that CD68 positive cells are increased during involution, suggesting that macrophages play a role in the poor prognosis of pregnancy associated breast cancer.
5. Ongoing and very promising development of 4 new mouse models for the study of pregnancy associated breast cancer;
 - a. Xenograft model in SCID mice with invasive human breast cancer MDA-MB-231 cells. In this model we have evidence that the involuting microenvironment increases primary tumor size.
 - b. Xenograft model in SCID mice for ductal carcinoma in situ using MCF10A DCIS cells. In this model we have evidence that the involuting microenvironment increases tumor incidence, shortens latency, increases tumor size (data not shown) and promotes local invasion.
 - c. Transgenic polyoma middle T model. Data from this model suggests that tumor latency is shortened, tumor size is promoted and lung metastasizes are enhanced in animals with pregnancies compared to virgin age-matched mice.
 - d. Bi-transgenic model from mating of polyoma middle t mice with Muc1 transgenic line. In this model, pregnancy has not been shown to enhance tumor size or shorten tumor latency, identifying pregnancy-induced Muc-1 as a possible mediator of the tumor promotion that occurs with pregnancy.

REPORTABLE OUTCOMES: Provide a list of reportable outcomes that have resulted from this research.

Manuscripts: None at present time.

Abstracts:

1. Borges V¹, Albrektsen G², Schedin P¹. *University of Colorado Denver*¹, Aurora, CO, USA, and *University of Bergen*², Bergen, Norway. **Identification of pregnancy-associated breast cancer (PABC) for investigation of the role of mammary gland involution in promoting metastasis in a Norwegian cohort of breast cancer cases.** Presented at the Department of Defense, Era of Hope Meeting, Baltimore, MD, June 25-28.
2. Schedin P, O'Brien J., Lyons T., Bell P., Man, Y., Wilson S., Lucia S., and Borges V. *University of Colorado Denver*, Aurora, CO, USA, 80010. **Role of mammary gland involution in promoting metastasis in pregnancy-associated breast cancer (PABC).** Presented at the Department of Defense, Era of Hope Meeting, Baltimore, MD, June 25-28.
3. Schedin P, Lyons T, O'Brien J, and Borges V. *University of Colorado Denver*, Aurora, CO, USA, 80010. **Common wound healing signature in murine and human involuting breast, implications for pregnancy-associated breast cancer (PABC).** Presented at the mammary Gland Biology Gordon conference, Barga Italy, June 1-6, 2008.
4. Schedin P, O'Brien J., Lyons T., Wilson S., Shroyer K., Lucia S., and Borges V. Department of Medical Oncology, *University of Colorado Denver, Anschutz Campus*, Aurora, CO, USA, 80010. **Role of mammary gland involution in promoting metastasis in pregnancy-associated breast cancer (PABC).** Presented at the Keystone meeting on Inflammation, Microenvironment and Cancer, Snowbird, UT, March 30-April 4, 2008.
5. O'Brien J., Lyons T., Wilson, S., Shroyer, K., Lucia, S., Borges V., and Schedin P. Department of Medical Oncology, *University of Colorado at Denver and Health Sciences Center*, Aurora, CO, USA, 80010. **Pro-Inflammatory Role of Tumor Microenvironment in Promoting Metastasis in Pregnancy-Associated Breast Cancer.** Presented at the AACR Special Conference, Advances in Breast Cancer Research, San Diego, CA, Oct 17-20, 2007.

Presentations

1. *Wound healing signature of mammary gland involution and pregnancy associated breast cancer*, Department of Physiology and Biophysics, University of Illinois Chicago, Oct 26, 2007.
2. *The inflammatory milieu permits metastasis in pregnancy-associated breast cancer*, Interdisciplinary Faculty of Toxicology program, Texas A&M University, Nov 13, 2007.
3. *Wound healing signature of mammary gland involution and pregnancy associated breast cancer*, Department of Molecular and Cellular Biology Seminar Program, Baylor College of Medicine, November 14, 2007.

4. *Identification of the Extracellular Matrix Proteomes Responsible for Breast Cancer Metastasis and Tumor Cell Dormancy*, Butcher Symposium on Genomics and Biotechnology, The Future of Biomedicine in Colorado, A Workshop of the Possible, Friday, November 16, 2007 Westin Hotel, Westminster, CO.
5. *The Inflammatory Milieu Permits Metastasis in Pregnancy-Associated Breast Cancer*, Laboratory of Cancer Biology and Genetics Seminar Series, NIH, Bethesda, MD, December 13, 2007.
6. *Pregnancy-Associated Breast Cancer*, Betty Ford Breast Cancer Treatment and Survivorship Symposium, Vail, CO, April 3-5, 2008.
7. *Role of mammary gland involution in promoting metastasis in pregnancy-associated breast cancer (PABC)*. Department of Defense, Era of Hope Meeting, Baltimore, MD, June 27, 2008.

Patents: None to date

Degrees obtained: None to date.

Tissue repositories: Human normal adjacent tissue representing pregnancy/lactation/involution cycle.

Infomatics/animal models: Four models under development; two that will permit the role of involution on breast cancer promotion to be investigate independent of the effects of pregnancy, and two models that will allow for the investigation of pregnancy and weaning-induced involution to be evaluated.

Funding applied for based on work supported by this award:

- 1) ACS Postdoctoral Fellowship awarded to Dr. Traci Lyons entitled ‘Mammary Gland Microenvironment in Breast Cancer Metastasis after Pregnancy’. This fellowship will support Dr. Lyons stipend during her training on the pregnancy-associated breast cancer project. The official start date of this fellowship is September 1, 2008.
- 2) DOD Predoctoral Fellowship awarded to Jenean O’Brien entitled ‘Determining the role of inflammation and macrophages during mammary gland involution and the subsequent effects on tumor promotion’. This fellowship will support Jenean O’Brien’s tuition and stipend as she trains on the pregnancy-associated breast cancer project. The official start date of this fellowship is June 15, 2008.

Training supported by this award:

- 1) Predoctoral Student, Jenean O’Brien of the Cancer Biology Program at UCD.
- 2) Postdoctoral Fellow, Dr. Traci Lyons.
- 3) Predoctoral MD/PhD student Jaime Fornetti of the Reproductive Sciences Program, UCD.

CONCLUSION: Previous data from our lab has identified mammary gland involution in rodents as being characterized by pro-inflammatory markers. These same markers have been linked to tumor progression, implicating mammary gland involution as a risk factor for breast cancer progression [2-8]. In our first year of funding on this Synergistic Idea Grant, we have been able to address whether mammary gland involution in women is also characterized by immune cell infiltrate. By performing chart extractions on young women with a history of breast biopsy, Dr. Borges has identified normal and normal adjacent breast tissue from women who have never been pregnant, are pregnant, lactating, weaning (breast tissue is actively involuting), or have fully regressed glands (parous). Using semi-quantitative IHC approaches, we have found that pro-inflammatory programs are activated during breast involution in women. This observation may account for the high rate of metastases associated with PABC. The rodent models for PABC under development in the lab will permit the investigation of mechanism of metastasis in PABC. The question of whether pregnancy and involution alter the subsequent development of breast cancer will be addressed by IHC using matched control and PABC cases obtained from our collaborator, Dr. Albrektsen in Norway [9-11]. These studies will commence in year 2 of funding.

"so what section" It can be argued that the tumor promotional contribution of mammary involution is a low level risk factor (RR range from ~1.1-1.3). However, it is an exceptionally high expressivity risk factor, affecting a large proportion of the female population. For example, if the conservative upper limit of 35 years of age for reproductive potential is chosen, an estimated 30,000 cases of breast cancer per year in the US may have a recent pregnancy as a negative prognostic feature. These women represent a readily identifiable high risk population that may benefit from prevention treatment. If successful, the pre-clinical results obtained from these proposed studies could translate to pre-clinical prevention studies and ultimately to the 'clinic' within 10 years. The 'clinic' is the obstetrician's office and the woman's treatment would be a preventive agent taken during the window of time when her breast tissue is in the 'drying out' period that follows delivery (if the woman does not nurse) or weaning (if she does).

REFERENCES:

1. Miller, R.R., Santner, S.J. Tait, L., and Dawson, P.J. , *MCF10DCIS.com xenograft model of human comedo ductal carcinoma in situ*. Journal of the National Cancer Institute, 2000. **92**: p. 1185-1186.
2. Bemis, L.T. and P. Schedin, *Reproductive state of rat mammary gland stroma modulates human breast cancer cell migration and invasion*. Cancer Res, 2000. **60**(13): p. 3414-8.
3. Schedin, P., et al., *Fibronectin fragments induce MMP activity in mouse mammary epithelial cells: evidence for a role in mammary tissue remodeling*. J Cell Sci, 2000. **113** (Pt 5): p. 795-806.
4. Schedin, P. and A. Elias, *Multistep tumorigenesis and the microenvironment*. Breast Cancer Res, 2004. **6**(2): p. 93-101.
5. Schedin, P., et al., *Mammary ECM composition and function are altered by reproductive state*. Mol Carcinog, 2004. **41**(4): p. 207-20.
6. McDaniel, S.M., Rumer, K.K., Biroc, S.L., Metz R.P., Singh, M., Porter W., and Schedin, P., *Remodeling of the Mammary Microenvironment after Lactation Promotes Breast Tumor Cell Metastasis*. Am J Pathol, 2006. **168**(2).
7. Schedin, P., *Pregnancy-associated breast cancer and metastasis*. Nat Rev Cancer, 2006. **6**(4): p. 281-291.
8. Schedin, P., et al., *Microenvironment of the involuting mammary gland mediates mammary cancer progression*. J Mammary Gland Biol Neoplasia, 2007. **12**(1): p. 71-82.
9. Albrektsen, G., et al., *Breast cancer incidence before age 55 in relation to parity and age at first and last births: a prospective study of one million Norwegian women*. Epidemiology, 1994. **5**(6): p. 604-11.
10. Albrektsen, G., et al., *Breast cancer risk by age at birth, time since birth and time intervals between births: exploring interaction effects*. Br J Cancer, 2005. **92**(1): p. 167-75.
11. Albrektsen, G., I. Heuch, and G. Kvale, *Further evidence of a dual effect of a completed pregnancy on breast cancer risk*. Cancer Causes Control, 1996. **7**(4): p. 487-8.