

Original Paper

# A Breast Tissue Protein Expression Profile Contributing to Early Parity-Induced Protection Against Breast Cancer

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## Key Words

Breast cancer • Parity • Prevention • Protein • Biomarkers

## Abstract

**Background/Aims:** Early parity reduces breast cancer risk, whereas, late parity and nulliparity increase breast cancer risk. Despite substantial efforts to understand the protective effects of early parity, the precise molecular circuitry responsible for these changes is not yet fully defined.

**Methods:** Here, we have conducted the first study assessing protein expression profiles in normal breast tissue of healthy early parous, late parous, and nulliparous women. Breast tissue biopsies were obtained from 132 healthy parous and nulliparous volunteers. These samples were subjected to global protein expression profiling and immunohistochemistry. GeneSpring and MetaCore bioinformatics analysis software were used to identify protein expression profiles associated with early parity (low risk) versus late/nulliparity (high risk). **Results:** Early parity reduces expression of key proteins involved in mitogenic signaling pathways in breast tissue through down regulation of EGFR1/3, ESR1, AKT1, ATF, Fos, and SRC. Early parity is also characterized by greater genomic stability and reduced tissue inflammation based on differential expression of aurora kinases, p53, RAD52, BRCA1, MAPKAPK-2, ATF-1, ICAM1, and NF-kappaB compared to late and nulli parity. **Conclusions:** Early parity reduces basal cell proliferation in breast tissue, which translates to enhanced genomic stability, reduced cellular stress/inflammation, and thus reduced breast cancer risk.

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## Introduction

Breast cancer is the most predominant cancer in women, currently affecting over 1.6 million women worldwide [1]. Despite improved detection and treatment strategies, breast cancer incidence rates continue to increase [2]. Moreover, metastatic breast cancer quickly acquires resistance to all therapies and there is currently no effective cure [3]. Current therapies for preventing and treating breast cancer also have many undesirable side-effects [3]. Thus, there has been a concerted effort to develop better strategies for prevention and treatment of breast cancer. In this regard, one primary area of study is parity-induced protection against breast cancer [4]. It is well-known that early pregnancy (before age 20) reduces breast cancer risk by as much as 50% [4-7]. In fact, early and multiple pregnancies further reduce a woman's risk of developing breast cancer by an additional 7% with each additional pregnancy [8]. In contrast, nulliparity or a first pregnancy later in life (after age 35) increases breast cancer risk [4]. A complete understanding of the natural and universal phenomenon of decreased breast cancer risk in early parous women will allow identification of biomarkers for predicting the risk of both breast cancer development and progression.

This phenomenon of parity-induced protection against breast cancer is also observed in rodent models where we and others have shown that both pregnancy and short-term treatment with pregnancy-level estrogen/progesterone protect against carcinogen-induced mammary cancer [9-14]. Both epidemiologic studies and *in vivo* rodent studies have revealed notable changes in the systemic hormone and growth factor profiles of parous females [9-11, 15-17]. These persistent systemic alterations have been shown to contribute significantly to parity-induced protection against breast cancer. However, many questions still remain regarding systemic versus local tissue-level changes that mediate the protective effects of early parity. Ongoing investigations suggest that changes in mammary gland responsiveness to hormones and growth-factors may play an important role in parity-mediated protection [18-23]. Indeed, substantial evidence from both epidemiologic and experimental studies indicate that the responsiveness of mammary epithelial cells to hormones and growth factors can change based on differences in receptor subtypes, co-activators, and hormone levels [4]. To date, studies reveal that estrogen receptor alpha (ER-alpha) and epidermal growth factor receptor (EGFR) expression decrease in parous mammary tissue, while ER-beta expression increases [4]. These observations are consistent with decreased estrogen sensitivity and increased cell maturation [4]. Unfortunately, the precise molecular circuitry involved in these changes is still not well-defined.

Recent studies in human subjects have assessed 1) genetic signatures of mammary tissue from parous and nulliparous women [22], [19]; and 2) genetic signatures of mammary tissue from women who have undergone reduction mammoplasty or breast biopsy for benign breast lesions [21]. Our study is therefore an invaluable addition to these prior studies because ours is the first large-scale study to compare the protein expression signatures of normal breast tissue from early parous, late parous, and nulliparous women. In this study, breast tissue biopsies were obtained from completely healthy women not undergoing breast-related surgery of any kind. With these samples, we have identified a protein expression signature unique to early parous (low risk) versus late/nulliparous (high risk) individuals. This unique protein expression signature significantly expands our understanding of tissue-specific changes that modify breast cancer risk.

## Materials and Methods

### *Antibodies and reagents*

Rabbit polyclonal anti-aurora A, rabbit monoclonal anti-S6K (E175), and rabbit polyclonal IGF2 were purchased from Abcam Inc (Cambridge, MA, USA). PhosphoExplorer antibody arrays and array assay kits were purchased from Full Moon Biosystems (Sunnyvale, CA, USA).

*Volunteers, sample collection and processing*

Healthy parous and nulliparous women between 40 and 60 yr. of age were recruited for this study at the Dr. Susan Love Research foundation in Santa Monica, CA (Table 1). Volunteer consent protocols for donating breast tissue by core needle biopsy and for the preservation of confidentiality (volunteer identities were not obtained) were reviewed and approved by both the Institutional Review Board of Texas Tech University Health Science Center in El Paso, TX and the Institutional Review Board of the Dr. Susan Love Research Foundation. Ultrasound guided breast tissue biopsies were obtained via core needle biopsy after application of local anesthetic. A small piece was fixed in formalin for histology and remaining tissue was snap frozen for further molecular analysis. Volunteers were divided into three groups: early parous (first full-term pregnancy  $\leq$  25 yr.), late parous (first full-term pregnancy  $\geq$  35 yr.) and nulliparous (never undergone a full-term pregnancy). All samples were collected at least 5 years after the last full-term pregnancy in all parous women. Only volunteers with a history of regular menses who were not on any medications or oral contraceptives within the last six months prior to sample collection were admitted to the study. Women with liver disease, renal disease, or endocrine problems were not included in the study.

*Global protein profiling*

Tissue proteomes of early, late and nulliparous women were probed for the expression of various proteins using the PhosphoExplorer antibody array (Full Moon BioSystems). Briefly lysis beads and extraction buffer were added to  $\sim$ 20 mg of tissue, vortexed for an hour on ice, and centrifuged (14,000 rpm / 20 min). The resulting supernatant was incubated at  $-80^{\circ}\text{C}$  for 4h followed by a second centrifugation step (14,000 rpm / 20 min). Clarified protein lysates were then applied to the antibody array, followed by fluorescence labeling with Cy3-streptavidin (GE Healthcare, Pittsburgh, PA, USA). Fluorescence intensity was measured using the Axon GenePix Array Scanner (Molecular Devices, Sunnyvale, CA, USA) and normalized to beta-actin expression levels for each array. Changes in protein expression were analyzed using GeneSpring software v. 12.5 (Agilent, Santa Clara, CA, USA).

*Immunohistochemistry of human breast tissue*

Individual proteins of interest were probed on formalin fixed, paraffin-embedded normal mammary tissue sections using standard immunohistochemistry techniques. Briefly, sections were deparaffinized and prepared by heat induced antigen retrieval with Trilogy (Cell Marque, Rocklin, CA, USA) at  $90^{\circ}\text{C}$  for 1 hr. The slides were washed with PBS and incubated 15 min with blocking buffer containing 1% FBS (Invitrogen, Grand Island, NY, USA) followed by PeroxFree blocking buffer for 5 min. Tissues were then incubated at  $4^{\circ}\text{C}$  overnight with IGFR2, P70S6K, or aurora kinase A (Abcam, CA, USA) antibodies as per manufacturer's recommendations. Slides were washed with PBS and overlaid with PolyScan HRP label (Cell Marque, Rocklin, CA, USA) for 30 min, washed again, and then overlaid with DAB chromogen (Cell Marque, Rocklin, CA, USA). Slides were incubated with DAB chromogen until proper color development was observed. Finally,

**Table 1.** Characteristics of study subjects

	<i>Parity Status</i>		
	Early parous	Late parous	Nulliparous
Age (mean $\pm$ SD)	51.5 $\pm$ 6.2 yr	50.5 $\pm$ 4.4 yr	51.1 $\pm$ 6.4 yr
Elapsed time since last pregnancy	$\geq$ 5 yr	$\geq$ 5 yr	$\geq$ 5 yr
Oral contraceptive use over prior 6 m	none	none	none
<i>Breast cancer history</i>			
Family	none	none	none
Personal	none	none	none
<i>Volunteer sample size</i>			
Total volunteers recruited (n)	44	44	44

tissue sections were rinsed in DI water, counterstained with hematoxylin, dehydrated with serial of ethanol baths followed by xylene and set with mounting media (Surgipath, Richmond, IL, USA). Representative IHC images were captured with the Nikon Eclipse 50i (Nikon Instruments Inc., USA).

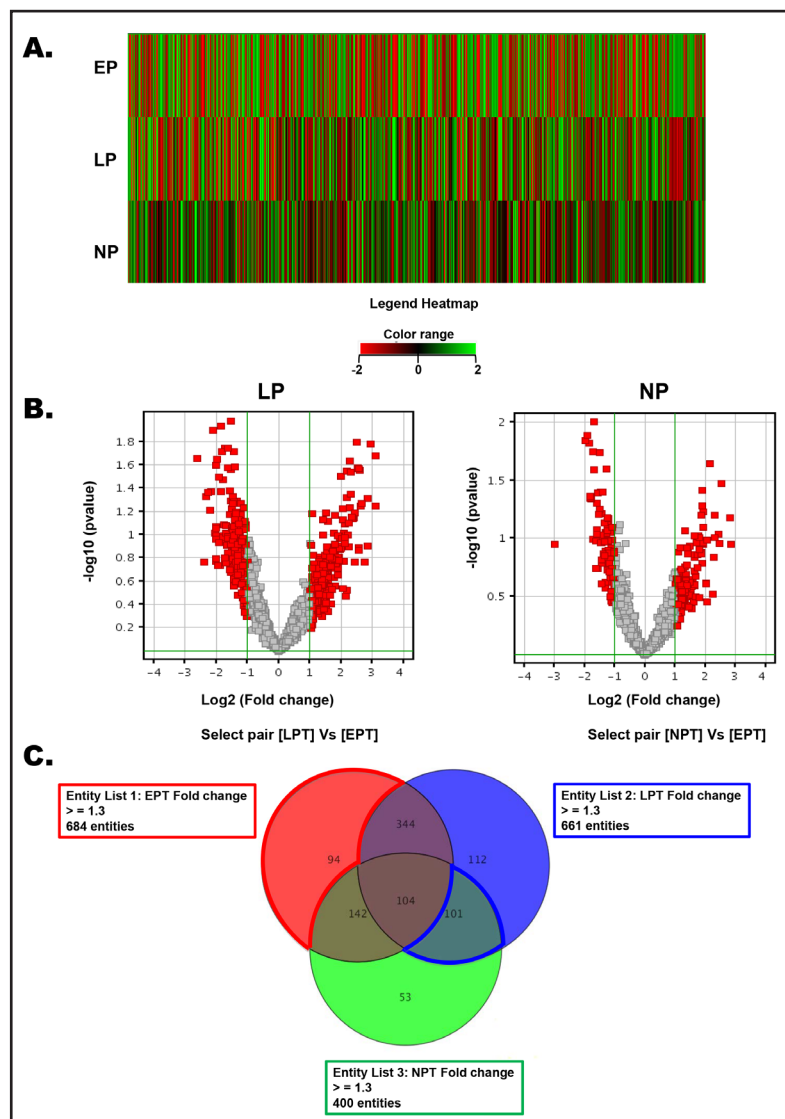
*Statistics*

Significant differences in protein expression between samples were assessed using un-paired Student's t-test. Asymptotic p-value computation and multiple correction testing were carried out using the Benjamini-Hochberg procedure. Volcano plots and the Venn diagram were generated using a fold change cutoff of  $\geq 1.3$  to identify proteins differentially expressed in early parous versus late and nulliparous groups.

**Results**

One hundred thirty two healthy volunteers were recruited for this study (Table 1). Global protein expression patterns of 1,318 phosphorylated and unphosphorylated proteins in breast tissue samples were assessed by protein antibody array. GeneSpring and MetaCore knowledge-based bioinformatics software were used to analyze the resulting data. Briefly,

**Fig. 1.** Tissue proteome analysis of late and nulliparous women compared to early parous women. GeneSpring bioinformatics analysis software was used to generate (A) protein expression heatmaps from the average expression of proteins in each sample group, (B) Volcano plots with a fold change cutoff of  $\geq 1.3$  and a p-value cutoff of  $\leq 0.05$  where red squares represent proteins that are differentially expressed in late/nulliparous women compared to early parous women, and (C) a Venn diagram with fold change and p-value cutoffs of  $\geq 1.3$  and  $\leq 0.05$ , respectively. Of particular interest in the Venn diagram are proteins with expression patterns unique to early parous subjects compared to late and nulliparous subjects (see highlighted slices). Early parous (EP, EPT), late parous (LP, LPT), nulliparous (NP, NPT).



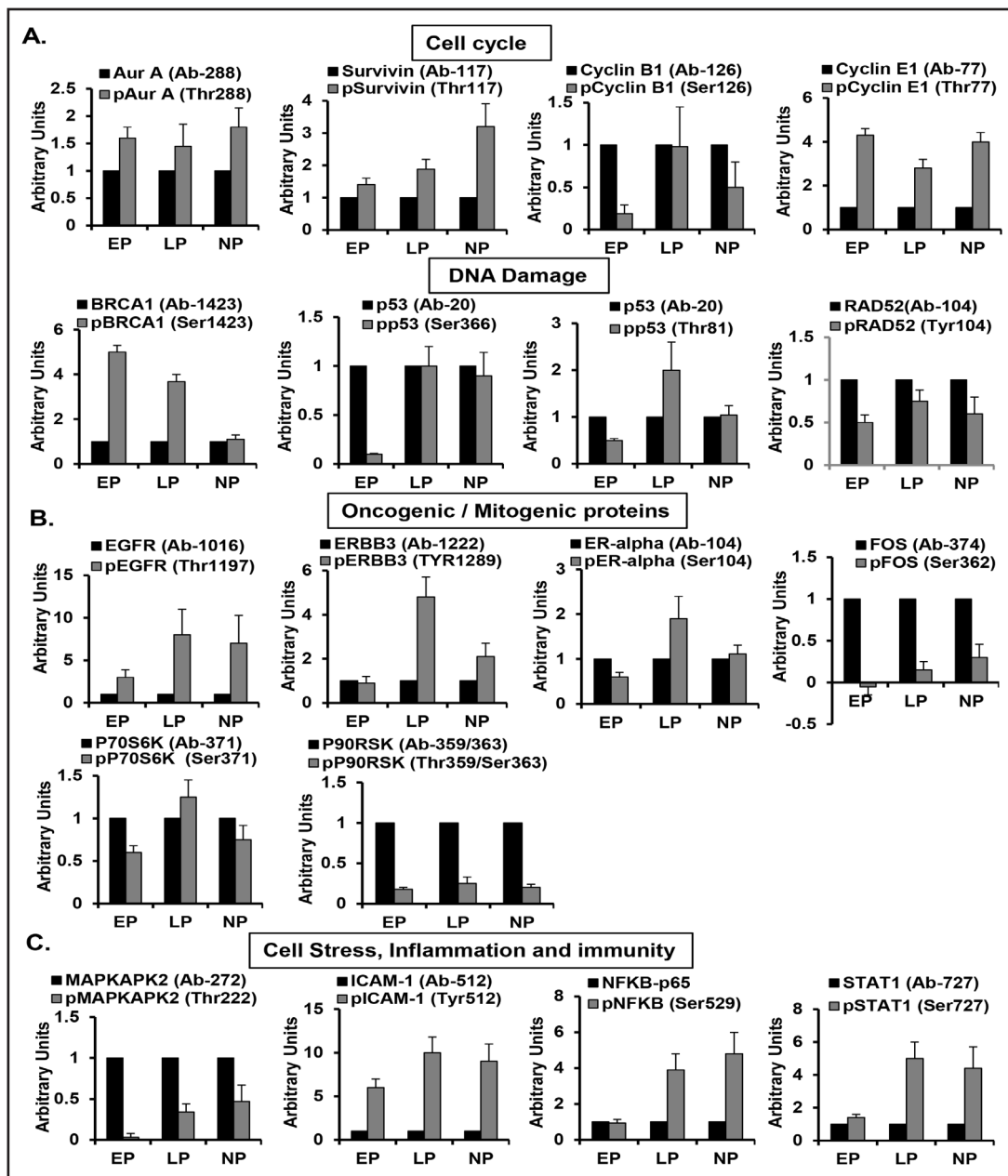
**Table 2.** Cell Cycle, DNA Damage Response and Apoptosis

Protein Name	Swiss Prot ID	Function	LP vs. EP (Fold Change)	NP vs. EP (Fold Change)
p53 (Phospho-Ser366)		Serine 366 phosphorylation by IKK-beta leads to TrCP1- and MDM2-dependent ubiquitination and degradation of p53.	4.9	3.6
Cyclin B1 (Phospho-Ser126)	P14635	Key role in regulation of cell cycle G2/M transition (mitosis regulation). Serine 126 phosphorylation required for CDC2 binding and kinase activity.	3.3	1.9
SMC1 (Phospho-Ser957)	Q14683	Regulates chromosome cohesion during the cell cycle and in DNA repair processes. Ser957 phosphorylation activates SMC1 and is required for S-phase checkpoint activity.	3.3	2.3
Survivin (Phospho-Thr117)	O15392	Inhibits apoptosis and also promotes cell proliferation. Facilitates centromere/midbody docking of AURKB during mitosis with the help of INCENP. Thr117 phosphorylation by AURKB prevents INCENP-Survivin interaction.	3.1	2.4
PEA-15 (Phospho-Ser116)	Q15121	Phosphoprotein with key roles in apoptosis inhibition and glucose transport. Blocks TNF-induced apoptosis by inhibiting caspase 8. Phosphorylation at Ser116 stabilizes protein and inhibits apoptosis.	7.0	3.1
Aurora Kinase A (Ab-288)	O14965	Mitosis regulation. Aids in progression of mitosis.	-2.5	-3.6
BRCA1 (Ab-1423)	P38398	Helps maintain genomic stability by facilitating DNA damage responses.	-1.8	-1.3
Caspase 9 (Ab-196)	P55211	Plays a role in apoptosis activation cascade. Enables apoptosis after DNA-damage in an ABL1/c-Abl dependent manner.	-1.5	-1.3
Cyclin E1 (Ab-77)	P24864	Regulates CDK2 activity in G1/S transition of the cell cycle.	-1.7	-1.4
p53 (Ab-20)	P04637	Cell cycle regulation, DNA damage responses, apoptosis.	-1.4	-1.3
p53 (Phospho-Thr81)		Threonine 81 phosphorylation stabilizes p53 in stress responses.	-1.7	-1.3
MDM2 (Ab-166)	Q00987	Ubiquitinates p53 targeting it for proteasomal degradation.	-1.9	-1.4
RAD52 (Phospho-Tyr104)	P43351	Double-stranded DNA break repair. Homologous recombination. Works together with RAD51. Tyrosine 104 phosphorylation activates RAD52 DNA repair functions.	-1.5	-1.5
PPAR-gamma (Ab-112)	P37231	Glucose homeostasis, cell cycle regulation. Mediates cell survival through inhibition of BAD and DAPK1.	-1.9	-1.4

raw data from each protein array was first normalized and subjected to a  $\pm 1.3$  fold-change cutoff and a p-value cutoff of  $\leq 0.05$ . Distinct differences in protein expression were observed for all three groups but there was a subset of proteins in late/nulliparous individuals that shared a common expression pattern distinct from that observed in early parous individuals (Fig. 1A – C). This particular protein expression profile is likely involved in early parity-mediated protection against breast cancer (Fig. 1C).

*Early parity alters expression of proteins involved in cell cycle regulation, DNA damage responses and apoptosis*

Several proteins involved in the regulation of cell cycle progression and apoptosis were notably altered by early parity (Table 2, Fig. 2A). Late/nulliparous women appear to have lower levels of total aurora kinase A (Table 2). This suggests that protein activation occurs at similar rates between the three groups, but total protein may be less stable or is translated less efficiently in late/nulliparous women. We also observe an increase in inhibitory phosphorylation of survivin, a key component of the chromosomal passenger complex (CPC) in late/nulliparous women (Table 2, Fig. 2A). In addition, the active form of cyclin B1 is highly up regulated in late/nulliparous women compared to early parous women, while cyclin E1 is down regulated in late/nulliparous women (Table 2, Fig. 2A). Active SMC1 (structural maintenance of chromosomes) is up regulated in late/nulliparous women, while BRCA1, p53, and RAD52 levels are down regulated or unstable in late/nulliparous women compared to early parous women (Table 2, Fig. 2A). Total BRCA1 levels are lower in late/nulliparous women (Table 2) as is the ratio of total-to-phosphorylated protein (Fig. 2A).



**Fig. 2.** Ratio of phosphorylated to total protein for key proteins that were differentially expressed in breast tissue according to parity status. Proteins of particular interest to parity-mediated protection against breast cancer were graphed as a ratio of phosphorylated to total protein. This reveals any potential differences in the ratio of total to active or inactive protein in late/nulliparous versus early parous individuals. (A) Proteins that play key roles in cell cycle progression and DNA damage responses, (B) proteins that are known to be proto-oncogenes or have mitogenic effects on breast epithelial cells, (C) proteins with a role in cell stress and inflammation/immune responses. Early parous (EP), late parous (LP), nulliparous (NP).

Similarly, we observe decreased threonine 81 phosphorylation and increased serine-366 phosphorylation of p53 in late/nulliparous women (Table 2, Fig. 2A), which renders p53 susceptible to ubiquitination and degradation [24, 25]. This indicates potentially impaired p53 activity in late/nulliparous individuals. The active form of RAD52 is also down regulated in late/nulliparous women although there is no difference in the ratio of active to total protein (Table 2, Fig. 2A). This indicates that protein activation likely occurs at similar

**Table 3.** Cell Proliferation, Cell Growth

Protein Name	Swiss Prot ID	Function	LP vs. EP (Fold Change)	NP vs. EP (Fold Change)
ABL1 (Phospho-Tyr204)	P00519	Cell growth and survival. Cytoskeletal reorganization, cell adhesion and motility, receptor endocytosis, autophagy, DNA damage response, apoptosis. Phosphorylation at this site likely activates the protein.	4.5	2.5
AKT1 (Phospho-Thr450)	P31749	Signal transduction, cell survival, cell growth, cell adhesion and motility, cytoskeletal remodeling, immunity, inflammation. Threonine 450 phosphorylation primes activation.	4.1	3.1
ATF-1 (Phospho-Ser63)		Phosphorylation at this site activates the protein in response to mitogens or cellular stress.	4.7	1.5
EGFR (Phospho-Tyr1197)	P00533	Epidermal growth factor receptor. Promotes cell proliferation and inhibits apoptosis. Autophosphorylated at tyrosine 1197 upon activation.	1.7	1.3
ELK-1 (Phospho-Ser383)	P19419	Transcription factor. ETS proto-oncogene. Regulates c-Fos expression. Involved in EGFR signaling, IGF-1 signaling, immunity, inflammation and development. Phosphorylated at this site by MAPK1 to activate transcriptional activity.	2.2	1.4
EPO-R (Phospho-Tyr368)	P19235	Erythropoietin growth factor signaling. Phosphorylation at this site associated with apoptosis inhibition. May affect the phenotypes of certain tumors.	1.5	1.6
Estrogen Receptor-alpha (Phospho-Ser104)	P03372	Nuclear hormone receptor. Female reproductive hormone signaling. Transcription factor. Important in cell proliferation and differentiation.	1.7	1.6
Ezrin (Phospho-Tyr353)		This phosphorylation site activates Ezrin in response to certain growth signals promoting cell survival, proliferation, and/or migration.	1.5	2.0
SRC (Phospho-Ser75)		This modification also leads to ubiquitination/degradation and cytoskeletal reorganization.	5.4	3.1
GSK3A (Phospho-Ser9)	P49840	Energy metabolism regulation, glycogen metabolism. Plays a role in cell division, proliferation, motility, and survival. Phosphorylates tau and presenilin-1.	3.2	2.7
PI3-kinase p85-subunit alpha/gamma (Phospho-Tyr467/Tyr199)	P27986	Regulatory subunit of the PI3K catalytic subunit. PI3K signaling. Tyrosine phosphorylation relieves inhibitory activity which allows PI3K signaling to proceed and enhances cell proliferation, survival, motility, and morphology.	3.3	1.5
MEK1 (Phospho-Ser298)	Q02750	Mitogen-activated protein kinase. Involved in various processes including proliferation, differentiation and transcriptional regulation. PAK1 primes MEK1 for activation by RAF-1 through serine 298 phosphorylation.	5.0	1.5
MER (Phospho-Tyr749)	Q12866	Receptor tyrosine kinase. Proto-oncogene. Cell survival, migration, differentiation, and clearance of apoptotic cells. Autophosphorylation at Tyr749 allows full activation.	2.5	5.8
Fos (Phospho-Ser362)	P01100	Signal transduction, cell proliferation and differentiation. Phosphorylation at this site activates c-Fos and promotes MAPK signaling.	4.2	2.2
HER3/ErbB3 (Ab-1222)	P21860	Neuregulin receptor. Epidermal growth factor signaling. Heterodimerization with family members possessing kinase activity results in cell proliferation, differentiation. Overexpressed in various cancers.	3.3	1.4
FAK (Phospho-Tyr407)		Phosphorylation at this site negatively regulates biologic and enzymatic activity.	2.7	2.9
FYN (Phospho-Tyr530)	P06241	SRC family proto-oncogene. PI3-kinase and integrin-mediated signaling. Cell growth, survival and adhesion. Phosphorylation at this site inhibits activity.	2.4	2.9
AKT1S1 (Ab-246)	Q96B36	AKT substrate. Subunit of mTORC1 which negatively regulates mTOR signaling when unphosphorylated. Phosphorylation by AKT relieves inhibitory effect on mTOR.	-1.9	-1.4
ATF-1 (Ab-63)	P18846	Activating transcription factor. Triggers cell proliferation and transformation.	-2.1	-1.8
FAK (Ab-910)	Q05397	Actin cytoskeleton reorganization. Cell migration adhesion and spreading. Growth factor receptor signaling.	-1.6	-1.4
IGF2R (Ab-2409)	P11717	Insulin-like growth factor 2 receptor. Transports various growth factors and phosphorylated lysosomal enzymes from the cell surface and Golgi to lysosomes. Putative tumor suppressor.	-1.5	-2.1
P70S6K (Ab-371)	P23443	Translation, cell metabolism, cell growth. Key downstream mediator of mTOR signaling.	-2.7	-3.2
P90RSK (Ab-359/363)	Q15418	Protein kinase downstream of ERK. Important in mitogen and cell stress responses. Modulates mTOR signaling and represses apoptosis.	-1.4	-1.3
SRC (Ab-529)	P12931	Non-receptor tyrosine kinase. Proto-oncogene. Phosphorylation at Ser-75 results in SRC kinase activation during mitosis in fibroblast and epithelial cells.	-2.3	-1.9

rates in all three groups, but perhaps the stability of the active protein is compromised in late/nulliparous women. Caspase 9—a key component for execution of several apoptotic pathways—is down regulated in late/nulliparous women (Table 2). Finally, elevated levels

of phosphoPEA-15 could decrease apoptosis by inhibiting caspase 8 and decreased levels of PPAR-gamma expression can mediate cell survival through inhibition of BAD and DAPK1 in late and nulliparous women. All together, these results suggest that compared to early parous women, late/nulliparous women may suffer from a higher level of genomic instability coupled with increased cell proliferation.

#### *Early parity alters expression of proteins involved in cell proliferation/cell growth*

Indeed, we have evidence that breast tissue from late/nulliparous women may have a higher rate of cell proliferation compared to breast tissue from early parous women. Many positive regulators of cell proliferation appear to be highly up regulated or activated in late/nulliparous breast tissue including PI3K/AKT, ABL, ATF, EGFR, HER3, ER-alpha, SRC, ELK-1, Ezrin, MEK1, MER, GSK3A and Fos (Table 3, Fig. 2B). However, there are also a few key proteins associated with increased cell proliferation that are down regulated or whose activity is inhibited in late/nulliparous women (Table 3). These include FAK, FYN, P70S6K and P90RSK. Not only are total FAK protein levels lower in late/nulliparous women, we also see up regulation of tyrosine 407 phosphorylation, which inhibits FAK function (Table 3). We further observe an increase in inhibitory phosphorylation of FYN, a SRC family kinase (Table 3). Total P70S6K and P90RSK protein levels are also lower in late/nulliparous women. Meanwhile, there is no difference in the ratio of active-to-total P90RSK in either group (Fig. 2B). Therefore, this protein expression pattern is still consistent with increased cell proliferation in late/nulliparous individuals.

It is also noteworthy that the putative tumor suppressor, IGF2R (insulin-like growth factor 2), is decreased in late/nulliparous breast tissue (Table 3). We know from prior studies that IGF2R expression is associated with decreased IGF2 because IGF2R acts as a molecular sink to control IGF2 levels [26]. Additional studies indicate that IGF2R also regulates other growth factors through its ability to bind mannose-6-phosphate tagged proteins and its function as a shuttle from endosomes to lysosomes [27]. Thus increased IGF2R would be expected to decrease cell proliferation, while decreased levels of IGF2R would be expected to increase cell proliferation in response to various growth factors [26].

#### *Early parity alters expression of proteins involved in cell stress and inflammation*

It is perhaps not surprising that late/nulliparous protein profiles also appear to be associated with increased cell stress and inflammation. If cell growth and proliferation are occurring at a higher rate than normal and this is in conjunction with increased genomic instability, then we can also expect higher levels of cellular stress and inflammation. A couple of proteins often activated by cellular stress are up regulated in late/nulliparous individuals compared to early parous individuals (Table 4). These are ATF-1 (pSer63) and MAPKAPK-2 (pThr222). Another series of proteins with proinflammatory functions are also differentially expressed in late/nulliparous women including ICAM-1, NF-kB p65 (pSer529), Ikb-beta (p-Thr19), IKK-beta (pTyr188), and STAT1 (pSer727) (Table 4). Several of these proteins also exhibit a clear trend for increased levels of active-to-total protein in late/nulliparous women (Fig. 2C). In a neuroblastoma cell line, both growth factor stimulus and cellular stress activated MAPKAPK-2, which in turn activated CREB and ATF-1 [28]. Also in cortical neurons, activating transcription factor 1 (ATF-1) protects from oxidative stress-induced apoptosis through a hypoxia-inducible pathway involving HIF-1, ATF-1/CREB, p21, and EPO (erythropoietin) [29]. Thus, it is interesting that here we see concurrent up regulation of active MAPKAPK-2, ATF-1 and erythropoietin receptor in late/nulliparous women (Table 3 and 4). It is intriguing to speculate that these proteins may play similar roles in breast epithelial cells where cell stress and/or growth factor stimulus would invoke this particular signaling pathway as a protective mechanism against oxidative and apoptotic stresses.

Further, the fact that ICAM-1 was significantly up regulated in breast tissue of late/nulliparous women (Table 4, Fig. 2C) is an indication of enhanced inflammation. ICAM-1 is normally expressed at low levels on epithelial cells and other cell types, but is quickly up regulated by inflammatory stimuli [30]. Moreover, ICAM-1 expression is up regulated in



**Table 4.** Inflammation and Immune Responses

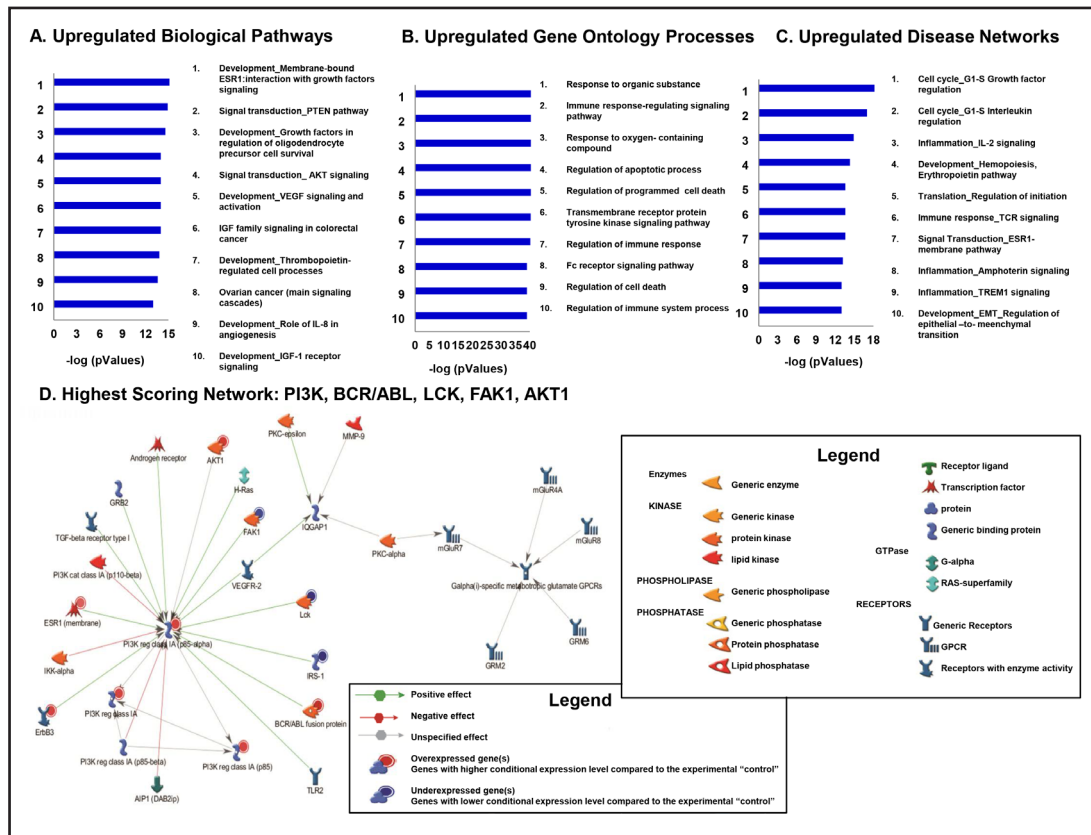
Protein Name	Swiss Prot ID	Function	LP vs. EP (Fold Change)	NP vs. EP (Fold Change)
ICAM-1 (Ab-512)	P05362	CD11a/CD11b integrin receptor. Key role in inflammation. Basal expression on immune cells, fibroblasts, endothelial cells, and epithelial cells. Upregulated in response to inflammation. Associated with various inflammatory immune responses and with epithelial tumorigenesis.	3.7	1.3
IκB-beta (Phospho-Thr19)	Q15653	Nuclear factor kappa-B inhibitors.	4.8	6.1
IKK-beta (Phospho-Tyr188)		Phosphorylation results in degradation and relieves NF-kappa-B inhibition.	4.0	1.7
MAPKAPK-2 (Phospho-Thr222)	P49137	Stress activated kinase. Cytokine production, cell migration and cell cycle control. Thr222 phosphorylation by p38 activates the protein.	6.9	8.5
STAT1 (Phospho-Ser727)	P42224	Mediates response to interferons, other cytokines and growth factors. Important role in regulating cell viability in response to different stimuli. Ser727 phosphorylation mediates IFN-gamma response or apoptosis induction when DNA damage occurs.	2.3	1.3
NFκB-p65 (Phospho-Ser529)	Q04206	Subunit of the nuclear factor-kappa-B transcription factor complex. Phosphorylation at this site activates the protein.	5.4	2.4
IKK-GAMMA (Ab-31)	Q9Y6K9	Nuclear factor kappa-B activator. Phosphorylates inhibitors of NF-kB. Mediates certain innate immune functions.	-1.8	-1.4
NFκB-p100/p52 (Ab-865)	Q04206	Subunit of the nuclear factor-kappa-B transcription factor complex. Among many functions, involved in regulating inflammation, immunity, cell growth and apoptosis.	-1.6	-12
LCK (Ab-393)	P06239	Tyrosine kinase. Src family member critical for selection and maturation of T cells.	-2.4	-1.5
LYN (Ab-507)	P07948	Non-receptor protein tyrosine kinase. Signaling intermediate. Regulation of innate and adaptive immunity. Important for proper immune system activation but also for immune self-tolerance.	-1.9	-1.4

human breast tumor samples compared to adjacent normal tissue and has been linked to enhanced metastatic properties in breast cancer [31]. We also see differential expression of several proteins in the NF-kappa B signaling pathway that indicate enhanced NF-kappa B signaling in late/nulliparous breast tissue (Table 4, Fig. 2D). A primary role for NF-kappa B in mediating innate immunity and inflammation has been appreciated for decades, and in many cases, has also been linked to cancer development and progression [32]. Further, there are also a few key proteins (IKK-gamma, LCK and LYN) associated with increased innate immunity, T cell maturation and inflammation that are down regulated in late/nulliparous women (Table 4). All together, these data indicate that compared to early parous breast tissue, late/nulliparous breast tissue may suffer from increased levels of proliferation, genomic instability, stress and inflammation.

These observations are in agreement with MetaCore knowledge-based bioinformatics analysis, which reveals strong associations with signaling pathways favoring cell growth/proliferation and immunity/inflammation (Fig. 3). Pathway enrichment analysis indicates that estrogen signaling and several other growth factor signaling pathways are enhanced in the late/nulliparous protein expression signature (Fig. 3A). Both gene ontology enrichment and process network analysis also suggest a strong connection with cell cycle regulation and various immune pathways—many of which enhance proinflammatory immunity—in late/nulliparous individuals (Fig. 3B, 3C). Again, when we subjected late/nulliparous protein expression profiles to MetaCore network analysis, we observed prominent contributions from pathways associated with cellular responses to growth factors (Table 5, Fig. 3D). The top scoring network reveals connections between steroid hormone signaling, epidermal growth factor signaling, and other pro-survival signaling pathways in late/nulliparous protein expression profiles (Fig. 3D).

#### *Immunohistochemistry Protein Array Validation*

In order to further validate the accuracy and reproducibility of these observations, we confirmed expression of key proteins via immunohistochemical (IHC) analysis of breast



**Fig. 3.** Enrichment analysis and network analysis of late/nulliparous versus early parous protein expression profiles. (A - C) Gene ontology and functional enrichment analysis was carried out to assess the principal molecular pathways associated with the unique protein expression pattern observed in late/nulliparous versus early parous individuals. Bar length is the negative logarithm of the enrichment p-value determined by MetaCore enrichment analysis. Bar length is therefore a direct measure of how significant the indicated association is. (A) Top ten scoring biological pathways determined by MetaCore enrichment analysis. (B) Top ten scoring gene ontology processes according to MetaCore gene ontology enrichment. (C) Top ten cellular processes associated with the late/nulliparous serum proteome according to MetaCore biomarker enrichment analysis. (D) Top scoring network of the AN algorithm for late/nulliparous versus early parous tissue protein profiles. Directional edges of the network diagram are marked with either green (activation) or red (inhibition) arrows.

epithelial tissue from early, late and nulliparous individuals. For this analysis, IGF2R, P70S6K, and aurora kinase A were chosen based on functional and/or statistical relevance to the study. IHC staining of mammary epithelial cells revealed that each of these proteins follows the same expression pattern as that observed in our protein array results (Fig. 4, Table 2 and 3). In agreement with the protein array results, these proteins are expressed at much higher levels in *early* parous breast epithelial cells compared to late/nulliparous breast epithelial cells (Fig. 4). Thus, the data from our protein array analysis appears to be accurate and reproducible. Moreover, it is significant that these protein expression patterns were faithfully reproduced specifically in breast epithelial cells (Fig. 4).

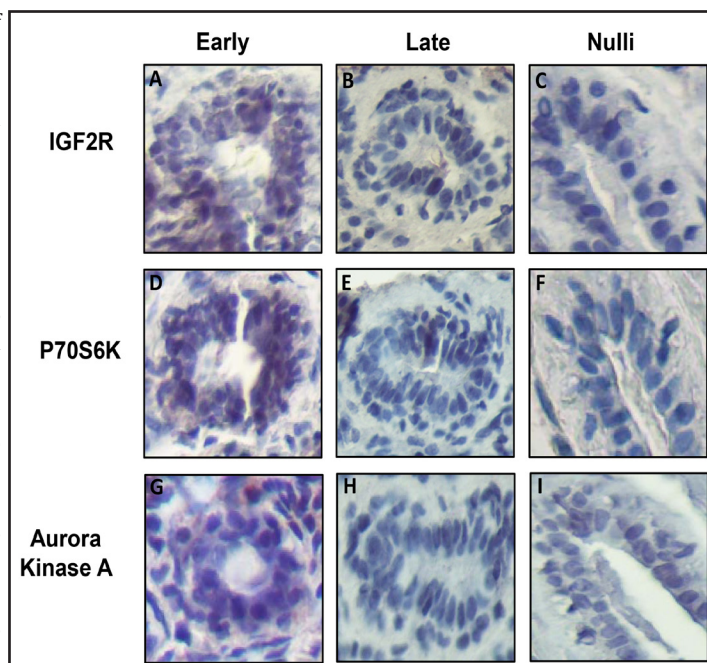
## Discussion

The purpose of this study was to identify a protein expression signature unique to early parous women that may be useful in predicting the risk of breast cancer development

**Table 5.** Top five networks for the analyze algorithm when comparing late/nulliparous versus early parous tissue protein profiles

No.	Network	GO Processes	Total nodes	Seed nodes	Canonical pathways	p-Value	zScore	gScore
1	PI3K reg class IA (p85-alpha), BCR/ABL fusion protein, Lck, FAK1, AKT1	fibroblast growth factor receptor signaling pathway (46.7%; 1.015e-20), regulation of phosphate metabolic process (80.0%; 4.372e-20), cellular response to fibroblast growth factor stimulus (46.7%; 5.374e-20), regulation of phosphorus metabolic process (80.0%; 5.505e-20), response to fibroblast growth factor stimulus (46.7%; 6.891e-20)	30	10	0	3.5E-22	49.54	49.54
2	p70 S6 kinase1, TAK1(MAP3K7), AKT(PKB), AKT1, Elk-1	cellular response to growth factor stimulus (65.5%; 6.234e-21), response to growth factor stimulus (65.5%; 1.880e-20), positive regulation of macromolecule biosynthetic process (79.3%; 3.147e-20), positive regulation of cellular biosynthetic process (79.3%; 2.302e-19), positive regulation of gene expression (75.9%; 2.692e-19)	30	9	0	1.4E-19	44.57	44.57
3	FKHR, AKT1, AKT(PKB), MDM2, EGFR	fibroblast growth factor receptor signaling pathway (53.3%; 9.229e-25), cellular response to growth factor stimulus (70.0%; 5.909e-24), cellular response to fibroblast growth factor stimulus (53.3%; 6.288e-24), response to fibroblast growth factor stimulus (53.3%; 8.374e-24), response to growth factor stimulus (70.0%; 2.013e-23)	30	9	0	1.4E-19	44.57	44.57
4	p53, STAT1, MDM2, IGF-2 receptor, Cyclin B	regulation of apoptotic process (65.5%; 3.546e-14), regulation of programmed cell death (65.5%; 4.167e-14), enzyme linked receptor protein signaling pathway (55.2%; 4.265e-14), regulation of cell death (65.5%; 9.136e-14), response to lipid (58.6%; 9.629e-14)	30	7	5	9.7E-15	35.22	41.47

**Fig. 4.** Immunohistochemistry of select proteins in early, late, and nulliparous breast epithelial tissue. Changes in protein expression detected by the PhosphoExplorer protein array were confirmed by immunohistochemistry staining of paraffin embedded tissue. Expression patterns of three representative proteins were assessed, including IGF2R (A, B, C), P70S6K (D, E, F), and Aurora Kinase A (G, H, I). Each panel shows the image of an individual mammary duct at 40X magnification. The first column of images is from early parous tissue samples, the second column from late parous tissue samples, and the last column from nulliparous tissue samples. Images are representative of three replicate experiments.



and progression in a diverse population of women. To our knowledge, this is the first large-scale study to assess global parity-induced changes *at the protein level* in normal human breast tissue. It is also our understanding that this is the first study to assess parity-related changes in normal human breast tissue stratified according to early and late parity, as well as, nulliparity. Many prior studies have assessed changes at the transcript level in parous versus nulliparous populations [4, 19, 21, 22]. The study design we have implemented here is critical for assessing changes in the mammary gland that truly play a role in parity-related breast cancer protection. There are many parity-related changes in breast tissue that may not play a role in protection against breast cancer or may even play an adverse role in breast cancer risk. In order to distinguish these effects from the true protective effects of parity we have compared protein expression changes in early parous women (low risk group) with protein expression changes commonly shared by both late and nulliparous women (high risk groups). Using this strategy, we successfully identified a protein expression signature unique to early parous women that is distinct from both late and nulliparous women.

This unique protein expression signature indicates that late/nulliparous breast tissue may suffer increased cell proliferation and stress, as well as, increased genomic instability and inflammation. These observations are in line with previous gene expression studies in both humans and rodents where the genomic profile of parous mammary tissue suggests a more mature and less proliferative state compared to non-parous counterparts [4, 19, 21, 22]. Indeed, a recent study reported increased levels of *Her2* (epidermal growth factor receptor 2) and *Esr1* mRNA in nulliparous versus early breast tissue [21]. Although we did not observe increased HER2 protein in this study, we did observe increased levels of two other proteins in the same family—EGFR (HER1) and HER3—in late/nulliparous individuals. Further, we also observed increased levels of ESR1 protein in late/nulliparous vs. early parous women. Thus, it appears that early parity represses human epidermal growth factor and estrogen signaling at both the transcript level [21] and the protein level—two key changes that likely contribute to reduced breast cancer risk in early parous women [4]. In line with increased growth factor and steroid hormone signaling in late/nulliparous women, we also found up regulation of several growth-promoting signaling networks in late/nulliparous breast tissue. These include PI3K/AKT, ATF, FOS and SRC signaling networks.

The concept that a higher degree of cell division can act as a “cancer promoter” for certain types of cancers surfaced in the late 1980’s based on epidemiologic evidence [33]. Genotoxic agents such as ionizing radiation or tobacco can by themselves directly cause genetic mutations that may result in malignant transformation. On the other hand, the constant DNA damage rate of a given cell and the resulting amount of irreparable DNA damage can be considered a direct function of that cell’s rate of division over time [33]. Thus, any agent or circumstance that enhances cell division would also be expected to promote malignant transformation over an extended period of time. This hypothesis is consistent with breast cancer etiology since hormone dependent breast cancers tend to develop much later in life and account for two thirds of all breast cancer cases [33]. Furthermore, genotoxic insult would be expected to have the most profound effect in a rapidly dividing cell population as opposed to a cell population where proliferation is much lower or even nonexistent, as in the case of Schwann cells which rarely divide and do not form cancers [33]. Therefore, whether increased rates of cell proliferation act alone or in concert with genotoxic insult, we can conclude that higher rates of cell division can significantly increase the risk of breast cancer in late/nulliparous populations.

In agreement with this, we also find evidence of increased genomic instability in late/nulliparous breast tissue. Aurora kinases A, B, and C are cell cycle proteins essential for appropriate chromosome alignment and segregation during cell division [34]. AurA (aurora kinase A) is required for microtubule formation/stabilization at the spindle pole to facilitate appropriate chromosome segregation during mitosis. Insufficient expression of AurA is associated with mitotic abnormalities that culminate in cytokinesis failure and polyploidy [34]. In particular, problems arise when aberrant expression of aurora kinases is accompanied by p53 deficiency as this can lead to aneuploidy or polyploidy [34]. Here, we have evidence that

p53 activity may be deficient in late/nulliparous women compared to early parous women. Even more concerning is the apparent deficiency in BRCA1 and RAD52 activity. Moreover, threonine-117 phosphorylation of survivin inhibits the association between INCENP and survivin, which can then prevent centromere/midbody docking of AurB during mitosis [34]. Given these observations, it is of particular concern that active cyclin B1 is up regulated in late/nulliparous women because cyclin B1 is essential for pushing the cells through mitosis. Cyclin E1 is expressed at lower levels, suggesting that there may be greater control at the S-phase of the cell cycle in late/nulliparous women. However, faster progression through mitosis is still of great concern as this is the final step in cell division. Therefore, combined p53/BRCA1/RAD52/aurora kinase deficiency and faster progression through mitosis may work together to increase genomic instability in late/nulliparous women. This would increase the chances of malignant transformation. Therefore, an important issue to address in future research is to determine the molecular events responsible for decreased levels/activity of these critical DNA-damage repair pathways. If these cells are indeed subject to a much greater level of genomic instability, it is likely that this is directly connected to the increased expression of proteins associated with greater cell stress and inflammation in late/nulliparous breast tissue.

In summary, we have shown that early parity is characterized by more efficient expression/activation of breast tumor suppressors p53, RAD52, and BRCA1. In addition, early parity significantly down regulates mitogenic EGFR, ESR1, and other pro-survival signaling pathways. Early parity may also be associated with less cellular stress and dampened inflammatory responses in the breast. This unique protein expression signature further expands our knowledge of how early parity may protect against breast cancer and will be invaluable for identification of breast cancer biomarkers and for development of superior breast cancer prevention/treatment strategies.

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## Disclosure Statement

The authors declare that there is no conflict of interest.

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