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## Life stage differences in mammary gland gene expression profile in non-human primates

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### Abstract

Breast cancer (BC) is the most common malignancy of women in the developed world. To better understand its pathogenesis, knowledge of normal breast development is crucial, as BC is the result of dysregulation of physiologic processes. The aim of this study was to investigate the impact of reproductive life stages on the transcriptional profile of the mammary gland in a primate model. Comparative transcriptomic analyses were carried out using breast tissues from 28 female

cynomolgus macaques (*Macaca fascicularis*) at the following life stages: prepubertal ( $n = 5$ ), adolescent ( $n = 4$ ), adult luteal ( $n = 5$ ), pregnant ( $n = 6$ ), lactating ( $n = 3$ ), and postmenopausal ( $n = 5$ ). Mammary gland RNA was hybridized to Affymetrix GeneChip<sup>®</sup> Rhesus Macaque Genome Arrays. Differential gene expression was analyzed using ANOVA and cluster analysis. Hierarchical cluster analysis revealed distinct separation of life stage groups. More than 2,225 differentially expressed mRNAs were identified. Gene families or pathways that changed across life stages included those related to estrogen and androgen (ESR1, PGR, TFF1, GREB1, AR, 17HSDB2, 17HSDB7, STS, HSD11B1, AKR1C4), prolactin (PRLR, ELF5, STAT5, CSN1S1), insulin-like growth factor signaling (IGF1, IGFBP1, IGFBP5), extracellular matrix (POSTN, TGFB1, COL5A2, COL12A1, FOXC1, LAMC1, PDG-FRA, TGFB2), and differentiation (CD24, CD29, CD44, CD61, ALDH1, BRCA1, FOXA1, POSTN, DICER1, LIG4, KLF4, NOTCH2, RIF1, BMP1A, TGFB2). Pregnancy and lactation displayed distinct patterns of gene expression. ESR1 and IGF1 were significantly higher in the adolescent compared to the adult animals, whereas differentiation pathways were overrepresented in adult animals and pregnancy-associated life stages. Few individual genes were distinctly different in postmenopausal animals. Our data demonstrate characteristic patterns of gene expression during breast development. Several of the pathways activated during pubertal development have been implicated in cancer development and metastasis, supporting the idea that other developmental markers may have application as biomarkers for BC.

## Keywords

Breast development; Gene microarray; Breast cancer; Stem cell; Estrogen; Progesterone

## Introduction

Major determinants of breast cancer (BC) risk include age, genetics, and reproductive history. Timing of reproductive milestones such as age at menarche and menopause, pregnancy, and lactation has an impact on breast development and thereby may alter the risk of developing BC later in life. Therefore, the study of breast development and differentiation across the lifespan may shed light on the origins of BC.

Mammary gland development may be separated into embryonic, adolescent, and adult phases, each of which is differentially regulated [1–4]. Embryonic branching of the breast is thought to occur without any hormonal requirement as it is not impaired in the absence of ER-alpha (ER $\alpha$ ), ER-beta (ER $\beta$ ), progesterone receptor (PGR), or the receptors for growth hormone (GH) or prolactin (PRLR). During adolescent branching, GH, insulin-like growth factor (IGF)-1, and ovarian estrogens as well as ER $\alpha$  are crucial; adult tertiary side-branching requires progesterone and its receptor [5].

All epithelial cells in the mammary gland are thought to originate from a common stem cell [6]. During differentiation from ER $\alpha$  negative stem cells to ER $\alpha$  positive progenitor cells and consecutively to basal- or luminal-restricted progenitors, these cells express different cell surface markers. Although unique stem cell markers have proven difficult to define, patterns of protein expression can distinguish mammary epithelial cells with stem-like or progenitor-like characteristics [7, 8].

As stem cells are undifferentiated and relatively resistant to apoptosis they may accrue DNA damage and mutations, making them susceptible to cancer initiation [9, 10]. The stem cell model of carcinogenesis proposes that BC originates in tissue stem or progenitor cells probably through deregulation of self-renewal pathways, resulting in BC cells with the

defining stem cell properties of self-renewal and differentiation. Self-renewal drives tumorigenesis, whereas differentiation contributes to tumor phenotypic heterogeneity [11].

To investigate the impact of reproductive stages and their respective characteristic hormonal profiles on mammary gene expression in the human breast, one would have to take breast biopsies from healthy girls and women without any exogenous hormonal exposure such as hormonal contraception, fertility drugs, or postmenopausal hormone therapy. This is practically and ethically impossible. A unique model for studying these questions is the monkey model. Female cynomolgus macaques (*Macaca fascicularis*) have distinctive reproductive similarities to women, including a 28-day menstrual cycle, comparable ovarian hormone patterns, and natural ovarian senescence. Macaques have >95% overall genetic sequence identity to humans, including key genes involved in BC susceptibility [12]. Human and macaque mammary glands are similar in terms of microanatomy and development, sex steroid receptor expression, responses to exogenous hormones, and the development of a heterogeneous spectrum of hyperplastic and neoplastic lesions with aging [13–15].

The aim of this study was to identify the impact of reproductive stages and their respective characteristic hormonal profiles on normal breast development by comparing gene expression patterns of the mammary gland from young animals (prepubertal and adolescent), adults (premenopausal in luteal cycle phase and postmenopausal), and animals in pregnancy-associated life stages (pregnancy and lactation) in cynomolgus monkeys. By gaining a better understanding of breast development, we also aimed to identify new biomarkers of BC that might proceed to new approaches in targeted therapies.

## Methods

### Animal characteristics and life stage selection

Healthy control female cynomolgus macaques (*Macaca fascicularis*) were imported from the Institut Pertanian Bogor (Bogor, Indonesia). Diets fed were nutritionally complete for non-human primates, free of soy isoflavones, and providing 42% of calories from fat in order to model the North American diet. Animals were housed in social groups of at least 4 animals. All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the Wake Forest University School of Medicine, which is accredited by the Association for the Advancement and Accreditation of Laboratory Animal Care. The animals (total  $n = 28$ ) were selected from four life stage groups: prepubertal ( $n = 5$ ), adolescent ( $n = 4$ ), adult luteal ( $n = 5$ ), pregnant ( $n = 6$ ), lactating ( $n = 3$ ), and postmenopausal ( $n = 5$ ). Ages were determined by dental eruption or known birth dates. Serum estradiol and progesterone levels were determined in the Yerkes Assay Services laboratory, Yerkes National Primate Research Center. Prepubertal and adolescent animals were of similar estimated age; the prepubertal stage was defined retrospectively, as referring to a sample taken more than 6 months prior to menarche. Adolescent animals were defined by menarche having occurred recently (within the preceding 6 months). Monkeys at adult luteal stage were in the luteal phase of the menstrual cycle, which was defined by endometrial and ovarian histology at necropsy. Pregnant monkeys were in the third trimester of gestation. Post-menopausal monkeys had undergone oophorectomy, which was performed at least 2.5 years prior to necropsy. Stages of breast development were confirmed by histopathology, using characteristics we have described previously [14, 16].

### Tissue collection

Mammary gland, including associated fat and connective tissues, was collected either by breast biopsy or at necropsy. All prepubertal and adolescent samples and one sample from a lactating animal were collected by biopsy. Necropsy samples were only taken from animals

from which they could be collected quickly (within 5 min of preplanned euthanasia). For biopsies, animals were anesthetized with ketamine (10–15 mg/kg) and buprenorphine (0.01 mg/kg), a 1.5-cm incision was made in the upper outer breast quadrant, and a small (0.4 gram) sample of mammary gland was removed. Biopsies were performed by an experienced veterinary surgeon. The incision was sutured, and the animals were monitored and given analgesia during recovery following IACUC-approved clinical procedures. Half of the biopsy sample was frozen; the other half was fixed at 4°C in 4% paraformaldehyde for 24 h, transferred to 70% ethanol, and then processed for histology using standard procedures. For necropsy samples, euthanasia was performed as part of unrelated IACUC-approved experimental protocols. Mammary tissues, ovaries, and uteri were removed, and fixed and frozen samples were collected as above. Each breast sample used for gene microarray analysis was evaluated by histology and mammary whole mount analysis to assure the presence of epithelial tissue with typical morphology for each developmental stage [17].

### Gene microarray assays

Total RNA was extracted from frozen mammary samples using Tri Reagent (Molecular Research Center, Cincinnati, OH), purified using an RNeasy Mini kit (Qiagen, Valencia, CA, USA), and quantified using a Nanodrop ND-100 UV–VIS spectrophotometer (NanoDrop, Wilmington, DE). RNA intactness and quality were confirmed using an Agilent 2100 Bioanalyzer (Wilmington, DE). Only samples with an RNA integrity number (RIN) greater than 8.0 were used for hybridization. One microgram of total RNA from each sample was labeled using the GeneChip One-Cycle Target Labeling kit (Affymetrix, Inc., Santa Clara, CA) following the manufacturer's protocol. RNA was then fragmented and hybridized to GeneChip Rhesus Macaque Genome Arrays (Affymetrix, Inc., Santa Clara, CA) for 17 h, prior to washing and scanning. Data were extracted from scanned images using GeneChip Operating Software (Affymetrix, Inc., Santa Clara, CA). RNA quality control and microarray assays were performed at Cogenics®, a Division of Clinical Data (Morrisville, NC; <http://www.cogenics.com>). One mammary gland sample from the prepubertal life stage group did not yield sufficient RNA for hybridization and was excluded, bringing the total number of arrays to 27 and the subset number of prepubertal animals to four.

### Quantitative RT-PCR

RNA was reverse-transcribed using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Transcript levels for targets related to estrogen, androgen, and prolactin metabolism were measured using quantitative real-time polymerase chain reaction (qRT-PCR). Cynomolgus macaque, rhesus macaque, or human ABI Taqman primer probe assays were used as targets, and samples were normalized to endogenous GAPDH and ACTB using cynomolgus macaque-specific primer probes. Stock macaque mammary tissues and macaque BC tumors were run in triplicate on each plate as external calibrators. Reactions were performed with ABI PRISM® 7500 Fast Sequence Detection System (Applied Biosystems), and relative expression was determined using the Ct method calculated by ABI Relative Quantification 7500 Software v2.0.1.

### Statistics

Microarray data analyses were performed using the GeneSifter software program (VizX Labs LLC, Seattle, WA, USA; <http://www.genesifter.net>). Intensity data were converted to a log<sub>2</sub> scale, screened for heterogeneity among samples, and evaluated using supervised analysis of variance (ANOVA) and pairwise comparisons between treatments. Principal components analysis (PCA), pattern navigation, and cluster analysis were performed on data subsets filtered by expression threshold. All P values were corrected for multiple comparisons using the Benjamini and Hochberg method ( $P_{adj}$ ) [18], which derives a false

discovery rate estimate from the raw P values [19]. Differences in gene numbers altered by each treatment were compared using a Fisher's exact test.

Quantitative RT-PCR data were analyzed using JMP (version 8.0.2, SAS Institute, Cary, NC). All relative expression data were evaluated for normality and homogeneity of variances among groups. Data were log-transformed and evaluated using ANOVA or the Kruskal–Wallis non-parametric test, then retransformed to original scale, and reported as fold change in prepubertal (control) group with 95% confidence interval. Multiple comparisons were made using Student's T-test for each pair, with two-tailed significance level of 0.05.

## Results

### Animal characteristics and tissue histology

Characteristics of animals in the study are presented in Table 1. Breast tissue of prepubertal animals consisted primarily of immature ducts and terminal end buds. Adult-type lobular units were present in all other life stages; pregnant animals were distinguished by marked lobular proliferation, and lactating animals by pronounced secretory activity. Representative histologic images of the breast tissue at each life stage are shown in Fig. 1, including immunohistochemistry for the proliferation marker Ki67.

### Global gene expression profiles

Principal components analysis and hierarchical clustering were initially used to evaluate mammary gland gene expression patterns across life stages (Fig. 2). Overall, 26,481 probe sets were detected at a quality  $>2$  and threshold fold change (FC)  $>1.5$ . Among these probes, PCA showed divergent vectors for juvenile animals (prepubertal and adolescent), pregnancy-associated life stages (pregnant and lactating), and adult animals (adult luteal and postmenopausal). Corresponding clustering dendrograms showed clear separation of all groups, with closer associations between the following groups: prepubertal and adolescent; pregnant and lactating; and adult luteal and postmenopausal.

Overall, 1,964 significantly differentially expressed probe sets were identified (ANOVA  $P_{\text{adj}} < 0.05$ ) for which  $FC > 1.2$ . The overall pattern of gene expression by PCA was similar regardless of the threshold for  $FC > 1.2$ , 2.0 and 3.0 at  $P_{\text{adj}} < 0.05$  for young animals. However, among the adult groups at higher FC, pregnant animals diverged from lactating animals and were more closely associated with adult luteal animals.

Most genes contributing to the difference between juvenile and adult animals were expressed in common by adult luteal, pregnant, and postmenopausal groups. However, most genes expressed by lactating animals were not shared by other groups. Between the two juvenile groups, no genes differed at a threshold of  $FC > 10$ , and there were only 137 differentially expressed genes at a threshold  $>1.8$  ( $P_{\text{adj}} < 0.05$ ). Adult groups showed similar overall directional patterns in gene expression changes, but each differed in the number of transcripts altered from prepubertal subjects and the magnitude of gene expression changes. For example, adult luteal and postmenopausal profiles resulted in 60 (37 up-regulated and 23 down-regulated) and 55 (36 up-regulated and 19 down-regulated) altered transcripts relative to the prepubertal profile with  $FC > 10$ , respectively. Even more differences in the number of altered transcripts were apparent when comparing pregnancy-associated life stages versus prepubertal profiles with  $FC > 10$  revealing 136 differences in pregnant animals (59 up-regulated and 77 down-regulated) and 437 differences in lactating animals (109 up-regulated and 328 down-regulated). Adult differences from the prepubertal group are shown graphically in Fig. 3.

Ten genes were regulated in common in all adult life stage groups and differed from prepubertal animals at threshold  $>10$  and  $P_{\text{adj}} < 0.05$  (Table 2). Among these differentially expressed genes, those associated with estrogen-regulated processes, proliferation, adhesion, and survival (*TFF1*, *GREB1*, *NEK10*, *LRRN3*, *PPM1K*, *PGR*, *IGFBP1*) decreased with advancing age, reaching their lowest levels during adult luteal phase (*IGFBP1*), pregnancy (*TFF1*, *NEK10*, *PPM1K*, *LRRN3*), lactation (*PGR*), and postmenopause (*GREB1*), respectively. Pairwise comparisons of gene expression between all groups revealed significant differences between prepubertal animals and all adult groups except postmenopausal. Among the four adult premenopausal life stage groups, significant differences were found for mammary *LRRN3*, *GREB1*, *PPM1K*, *NEK10*, and *PGR* gene expression ( $P_{\text{adj}} = 0.05$ ). *LRRN3* expression was significantly lower in pregnant than in postmenopausal breast tissue. *PGR* expression was significantly lower in lactating than in pregnant and adult luteal breast tissue. *GREB1* expression was lower in lactating animals than in pregnant animals. Conversely, *PPM1K* and *NEK10* gene expression were significantly lower in pregnant than in lactating breast tissue.

In contrast, the expression of three genes associated with glandular differentiation (*CSN1S1*, *IGHG1*, and *ELF5*) was significantly increased with advancing age when compared with prepubertal life stage, reaching their maximum during the luteal phase of the menstrual cycle (*IGHG1*) or lactation (*CSN1S1*, *ELF5*), respectively ( $P_{\text{adj}} = 0.05$ ). Pairwise comparisons of differentiation gene expression revealed a significant difference between prepubertal and adult pre-menopausal or pregnancy-associated life stages ( $P_{\text{adj}} = 0.05$ ), but again, not for postmenopausal subjects. The differentiation markers *CSN1S1* and *ELF5* peaked during lactation, differing from all other groups including pregnant mammary gland ( $P_{\text{adj}} = 0.05$ ). Postmenopausal mammary *ELF5* expression was significantly decreased when compared with the adult luteal and pregnancy-associated life stages ( $P_{\text{adj}} = 0.05$ ).

A secondary goal of this study was to identify signaling pathways that are significantly altered across life stages (threshold  $> 1.5$ ; ANOVA  $P_{\text{adj}} < 0.00001$ ). Overall, 4,962 differentially regulated genes covering 10 KEGG pathways and 23 relevant gene ontology cohorts were identified. We focused on pathways related to androgen and estrogen metabolism, mammary stem cells, and profiles relevant for BC development.

### Sex steroid receptors and steroid metabolism

Gene expression differences by array for the major receptors of the estrogen, progesterone, prolactin, and androgen (*ESR1*, *PGRB*, *PRLR*, *AR*), and key enzymes in steroid metabolism are shown in Table 3. *ESR1* and *PGRB* decreased in the transition from prepuberty to adolescence, and *AR* gene expression was maximal during adolescence after which it significantly decreased ( $P_{\text{adj}} = 0.05$ ). *ESR1* expression had its minimum during postmenopause, whereas *AR* and most strikingly *PGRB* expression were lowest during lactation. Lower gene expression for *ESR1*, *PGRB*, and *AR* during lactation was significant relative to other life stages ( $P_{\text{adj}} = 0.05$ ). *PGRB* was less than  $<1/100$ th of prepubertal levels during lactation. Expression of *PRLR* significantly increased with maturity in comparison with the prepubertal life stage, reaching its maximum during pregnancy where it was significantly higher than in lactating and postmenopausal breast ( $P_{\text{adj}} = 0.05$ ).

Five genes involved in C21, C19, and C18 steroid formation were differently expressed relative to prepubertal gene profiles (*AKR1C4*, *STS*, *HSD11B1*, *HSD17B2*, and *HSD17B7*; Table 3). *AKR1C4* and *STS* decreased during the pubertal transition and were lower in all adult groups. Expression of three genes (*STS*, *HSD11B1*, and *HSD17B7*) coding for enzymes associated with the formation of more potent steroid hormones was significantly decreased with maturity when compared with prepubertal animals, reaching their minimum during pregnancy (*HSD11B1*, *HSD17B7*) and lactation (*STS*), respectively ( $P_{\text{adj}} = 0.05$ ).

However, for these genes, postmenopausal mammary gene expression significantly increased to levels comparable to adolescent and adult luteal life stages for *STS*, *HSD11B1*, and *HSD17B7* ( $P_{\text{adj}} = 0.05$ ). Expression of two genes (*HSD17B2* and *AKR1C4*) coding for enzymes associated with steroid hormone inactivation to less-potent metabolites was either significantly increased (*HSD17B2*) or decreased (*AKR1C4*) with advancing age when compared with prepubertal life stage, respectively ( $P_{\text{adj}} = 0.05$ ; Table 3).

### Markers of stem cells and differentiation

Life stage effects on genes related to mammary stem cells and differentiation were evaluated using gene ontology analysis (stem cell division  $n = 9$ ; stem cell differentiation  $n = 33$ ). Genes differentially regulated at  $FC > 1.2$  versus prepubertal ( $P_{\text{adj}} = 0.00001$ ) were initially used for this analysis ( $n = 4,955$ ). Herein, 8 genes were found to be statistically significant ( $P = 0.001$ ) for stem cell regulation (*DICER1*, *IGF1*, *LIG4*, *KLF4*, *NOTCH2*, *RIF1*, *BMPR1A*, and *TGFB2*). In addition, we analyzed our data set for gene expression patterns of stem cell markers presented by LaMarca and Rosen (*ELF5*, *CD29*, *CD24*, *CD61*, *CD44*, *ALDH1*, *BRCA1*, *GATA3*, *FOXA1*, *STAT5*, and *POSTN*) [6], and we also included the proliferation marker MKI67 in this category (Table 4).

A distinct pattern of decreased gene expression for putative stem cell markers and regulators of differentiation was seen during pregnancy or lactation when compared to juvenile and adult animals; diminished expression was seen for *DICER1*, *RIF1*, *BMPR1A*, *TGFB2*, *LIG4*, *KLF4*, *NOTCH2*, *CD44*, *FOXA1*, and *CD29* ( $P_{\text{adj}} = 0.05$ ). In contrast, mammary gene expression of the differentiation and stem cell markers *ELF5*, *CD24*, *STAT5*, and *ALDH1* increased with advancing age peaking during pregnancy or lactation ( $P_{\text{adj}} = 0.05$ ). Other potential stem cell markers presented a heterogeneous gene expression profile across life stages (*KLF4*, *POSTN*, *NOTCH2*, *IGF1*, *BRCA1*, *LIG4*, and *CD61*) with an increase during puberty followed by a decrease during pregnancy-associated life stages (*KLF4*, *POSTN*, *NOTCH2*, *IGF1*, *BRCA1*, and *LIG4*) and postmenopause (*POSTN*, *IGF1*, and *BRCA1*;  $P_{\text{adj}} = 0.05$ ). MKI67 gene expression remained relatively stable across life stages.

The luminal progenitor cell population is characterized by the cell surface marker *CD29<sup>lo</sup> CD24<sup>+</sup> CD61<sup>+</sup>* and the differentiated luminal cell population by *CD29<sup>lo</sup> CD24<sup>+</sup> CD61<sup>-</sup>*. Pairwise comparisons revealed a significant increase in *CD24* gene expression in lactating mammary gland in comparison with other life stages, whereas *CD29* gene expression was significantly lower in pregnant and lactating mammary gland compared to young animals ( $P_{\text{adj}} = 0.05$ ). *CD61* gene expression was significantly higher in adult luteal and lactating and lower in pregnant and postmenopausal mammary gland in comparison with prepubertal subjects ( $P_{\text{adj}} = 0.05$ ). Expression of four genes associated with cell differentiation, repair mechanisms, and apoptosis (*DICER1*, *RIF1*, *BMPR1A*, and *TGFB2*) was significantly decreased with advancing age when compared with prepubertal animals ( $P_{\text{adj}} = 0.05$ ).

Expression of six genes associated with cell differentiation was significantly increased during adult life stages when compared with juvenile animals (*LIG4*, *KLF4*, *NOTCH2*, *ELF5*, *STAT5*, *ALDH1*;  $P_{\text{adj}} = 0.05$ ). Within adult premenopausal life stages, no significant differences in expression of *DICER1*, *IGF1*, *LIG4*, *KLF4*, *NOTCH2*, *RIF1*, *BMPR1A*, *TGFB2*, *STAT5*, or *ALDH1* were observed. Surprisingly, postmenopausal mammary gene expression did not differ significantly from the adolescent expression pattern, with the exception of *IGF1* gene expression that was significantly higher during adolescence than in any other life stage examined ( $P_{\text{adj}} = 0.05$ ). Within pregnancy-associated life stages, *DICER1*, *IGF1*, *LIG4*, *NOTCH2*, *STAT5*, *CD29*, *BRCA1*, and *POSTN* gene expression was significantly lower in breast tissue of lactating animals relative to pregnant animals ( $P_{\text{adj}} = 0.05$ ). The opposite was true for *CD24* and *CD61* ( $P_{\text{adj}} = 0.05$ ). Genes *DICER1*, *IGF1*, *LIG4*, *NOTCH2*, *KLF4*, *RIF1*, *BMPR1A*, *TGFB2*, *CD29*, *CD44*, *FOXA1*, *BRCA1*, and

POSTN exhibited lower expression in lactating compared to postmenopausal breast tissue ( $P_{\text{adj}} = 0.05$ ). The opposite was true for ELF5, CD24, STAT5, ALDH1, and CD61 gene expression ( $P_{\text{adj}} = 0.05$ ).

### Extracellular matrix signaling

Life stage effects on genes related to extracellular matrix signaling were evaluated using gene ontology analysis (extracellular matrix organization  $n = 179$ ). Genes differentially regulated at  $FC > 1.2$  versus prepubertal ( $P_{\text{adj}} = 0.00001$ ) were initially used for this analysis ( $n = 42$ ). We selected eight significantly differently regulated genes (Table 5).

Overall, gene expression (*POSTN*, *TGFB1*, *COL5A2*, *COL12A1*, *LAMC1*, *PDGFRA*, *TGFB2*) was decreased during all life stages in comparison with prepubertal animals reaching a nadir during lactation (*TGFB1*, *PDGFRA*, *LAMC1*, *COL12A1*, *COL5A2*;  $P_{\text{adj}} = 0.05$ ). *POSTN* in particular was expressed most abundantly in juveniles, with a tenfold lower expression in cycling or postmenopausal adults and a 30- to 90-fold lower expression in pregnant and lactating animals, respectively. However, there was a significant initial increase in *TGFB1*, *POSTN*, and *COL5A2* gene expression during adolescence ( $P_{\text{adj}} = 0.05$ ). In contrast, *FOXC1* was significantly up-regulated in adulthood in comparison with prepuberty ( $P_{\text{adj}} = 0.05$ ), with lower expression in lactation.

### Breast cancer–associated genes

Next, we selected genes that have been associated with BC development, described in the 70-gene Amsterdam signature that forms the basis for the MammaPrint<sup>®</sup> assay [20]. Out of 50 genes represented in the MammaPrint<sup>®</sup> assay, 21 probes were significantly differently regulated at  $P_{\text{adj}} = 0.05$  in our data set, representing six genes (Table 6).

In general, mammary expression of *SERF1A*, *GTM3*, and *PECI* genes decreased significantly with advancing age in comparison with prepubertal life stage, reaching their minimum during pregnancy (*PECI*) and lactation (*SERF1A*, *GTM3*), respectively ( $P_{\text{adj}} = 0.05$ ).

However, gene expression of *IGFBP5* and *TGFB3* revealed a significant increase during adolescence, followed by a similar significant decrease in advanced life stages, also reaching a minimum during lactation ( $P_{\text{adj}} = 0.05$ ). In contrast, *CENPA* gene expression was significantly increased in adolescent, adult luteal, and pregnant mammary glands when compared with prepubertal subjects and was only significantly decreased during lactation and postmenopause ( $P_{\text{adj}} = 0.05$ ). Lactating mammary gland presented a distinct gene expression pattern since pairwise analysis revealed a significant difference between lactating and young and adult life stages for *SERF1A*, *IGFBP5*, *GTM3*, and *TGFB3*, respectively ( $P_{\text{adj}} = 0.05$ ). There was a significant difference between lactating and pregnant mammary gene expression for *GSTM3*, *IGFBP5*, *TGFB3*, and *CENPA* ( $P_{\text{adj}} = 0.05$ ).

### Quantitative RT-PCR

Quantitative RT-PCR was also used to assess expression levels of 14 key genes associated with estrogen, androgen, and prolactin signaling and metabolism, which had shown differential expression by microarray. Six of these showed significant fold change in expression in adulthood compared to prepuberty (Fig. 4). The genes were *MKI67*, *PRLR*, *AR*, *SULT*, *HSD17B1*, and *HSD17B2*. We found that expression profile of these genes among different life stages was similar to that shown with microarray. Consistent with our microarray results, *MKI67* and *PRLR* were up-regulated during adulthood and pregnancy. Expression of *AR* was down-regulated in pregnancy and lactation. Expression of *SULT* mRNA, encoding for the enzyme that sulfates and thereby deactivates estrone, was



diminished during pregnancy, relative to other groups. Expression of *HSD17B1*, which encodes a major estrogen biosynthetic enzyme converting estrone to estradiol, was highest in prepubertal animals. Conversely, mRNA for the enzyme catalyzing the reverse reaction (*HSD17B2*) was more abundant in adult life stages.

## Discussion

Our data demonstrate distinct life stage-specific patterns of gene expression during breast development. Simplified examples of expression patterns are shown in Fig. 5. With respect to mammary development during puberty, our findings support previous observations that GH operating via IGF-1, ovarian estrogens, and the respective receptors are crucial for normal breast development [2].

Estrogen-regulated genes such as *ESR1* and *IGF1* were found to be up-regulated during adolescence. The increase in *IGF1* was not accompanied by an increase in *IGFBP1* that would hamper its proliferative activity by protein binding. Other estrogen-regulated genes such as *GREB1*, *TFF1*, and *PGR(B)* were elevated during pubertal stages and decreased with advancing age. *GREB1* is an ER target gene and ER coactivator that regulates estrogen-induced proliferation in BC cells. It is overexpressed in ER -positive BC by 3.5-fold compared to ER -negative BC [21]. TFFs are associated with mucin-secreting epithelial cells and contribute to mucosal defense and healing. *TFF1* is expressed in approximately 50% of human breast tumors and enhances anchorage-independent growth, increased cell migration, and invasion [22]. As an estrogen-induced protein, it indicates likely responsiveness to endocrine treatment [23]. Progesterone via its receptor *PGR* is essential for mammary lobuloalveolar development. In virgin mammary gland, the A subtype of *PGR* dominates [24]. The *PGR B* signaling pathway shares regulatory cross-talk with other pathways such as *RANKL* [25]. The ratio of *PGR* isoforms may have a regulatory effect during development [26, 27]. *PRLR* expression has implications beyond prolactin per se; placental lactogen and primate growth hormones bind the *PRLR* [28, 29], which results in the activation of various signaling pathways including *Jak2/Stat5* [30, 31], *Shc/Grb2/Ras/Raf/Mek/MapK* [32–34], and *PKB/PI3 K* [35–37].

Pregnancy-associated life stages markedly differed from other life stages. Prolactin is a major driver of development during pregnancy both directly and through stimulation of ovarian progesterone production. These hormones induce rapid and global proliferation of epithelial cells within the ductal epithelium and developing alveoli. During the second half of pregnancy, the cells of the alveoli differentiate and polarize to form the secretory alveolar epithelium, capable of milk production and secretion during lactation. Withdrawal of ovarian and placental progesterone brings about the onset of secretory activation [38]. Accordingly, *PRLR* gene expression reached its maximum during pregnancy and remained high during lactation in our study. Similarly, gene expression of *CSN1S1*, one of the predominant phosphoproteins in milk, tremendously increased during lactation. However, production of caseins is also stimulated by cortisol [37]. Enzyme *HSD11B1* catalyzes the reduction of cortisone to active cortisol, and not surprisingly, we found that *HSD11B1* gene expression paralleled the increased production of milk proteins.

During postmenopause, expression of various genes came back to adult luteal level (*STS*, *HSD11B1*, *HSD17B7*, *HSD17B2*, *TFF1*, *GREB1*, *CSN1S1*, *IGFBP1*, *NEK10*, *IGHG1*, *LRRN3*, *PPM1K*, *PGR*, *ESR1*, *PRLR*, *AR*). However, since the animals in this study were imported from abroad where they had lived in a breeding colony, it is likely that they had been pregnant before. Therefore, the gene “thumb print” of past pregnancy may be still present in our adult luteal subgroup.

AR gene expression decreased across life stage. AR rapidly modulates the expression of genes involved in proliferation and differentiation. Only recently, short-term androgen treatment has been shown to diminish the estrogen-induced proliferative response of the breast in rhesus monkeys [39, 40]. Furthermore, there is emerging evidence that the androgen-signaling pathway plays a critical role in breast carcinogenesis, independent of ER. In humans, although hyperandrogenemia is thought to be a risk factor for BC, expression of AR in BC is associated with lower tumor burden and favorable differentiation [41].

Breast tissue and mammary cancer cells possess the enzymatic systems necessary for local biosynthesis of steroid hormones, including enzymes involved in both the activation (STS, HSD11B1, HSD17B7) and inactivation (HSD17B2, AKR1C4) of hormones. The increase in ovarian serum estrogens during puberty and even more during pregnancy was accompanied by a decrease in mammary STS and HSD17B7 and an increase in HSD17B2 favouring the formation of estrone, estrone sulfate, and estriol. This control mechanism may protect the mammary gland from excessive estrogenic exposure. Indeed, HSD17B2 has been shown to be the predominant HSD17B subtype in normal breast tissue, whereas in cancerous tissue, the ratio of HSD17B1 to HSD17B2 changes, leading to a higher amount of locally formed potent estradiol [42]. Similarly, STS activity is significantly higher in cancerous in comparison with normal breast tissue [43].

There is evidence for a hierarchical model in which all types of epithelial cells in the mammary gland originate from a common multipotent stem cell [6]. In this model, ER<sup>-</sup> negative stem cells (CD24<sup>+</sup>/CD29<sup>hi</sup>) undergo asymmetric division to give rise to undifferentiated, ER<sup>-</sup> positive progenitor cells. These multipotent progenitor cells may also differentiate into basal-restricted or luminal-restricted progenitors and alveolar-restricted lineages (CD61<sup>+</sup>). During pregnancy, prolactin-mediated GATA3 may contribute to alveolar cell development, whereas ELF5, also a target of prolactin signaling, establishes the secretory alveolar lineage [44].

Across life stages, we found a significant increase in CD24 and ALDH1 gene expression, which was highest during lactation; this seems to indicate an amplification of uncommitted progenitor cells during breast development but may also indicate a lack of specificity for these putative stem cell markers in the context of the mature breast. Further differentiation to CD61-expressing luminal progenitors mainly occurred during adult luteal and lactating life stages. We also found an increase in ELF5 gene expression across life stages, reaching a maximum during adult luteal and lactating mammary gland, respectively, thus paralleling CD61 and PRLR gene expression. As mentioned above, GATA3 regulates mammary cell fate at multiple time points throughout mammary gland development including embryonic stage. Thus, it is not surprising that we did not find a significant impact of the life stages examined when using prepubertal animals as a reference of comparison (data not shown).

The association of ALDH1 and BRCA1, which has well-established roles in DNA repair and chromosome stability, has been investigated by Wicha et al. [45]. They suggest that BRCA1 plays a role in the differentiation of ALDH1-positive/ER-negative stem/progenitor cells into ER<sup>-</sup> positive luminal epithelial cells. A loss of BRCA1 function may therefore cause a block in epithelial cell differentiation and the expansion of undifferentiated, ER-negative stem cells. BRCA1 expression was highest early in life and reduced in pregnancy, lactation, and postmenopausal phases, with a nadir during lactation.

There is a variety of stem cell markers generally associated with cell differentiation, repair mechanism, and apoptosis, which displayed different gene expression patterns across life stages. The majority of stem cell markers analyzed revealed a decrease in gene expression

immediately with the transition into puberty (DICER1, RIF1, BMPR1A, TGFB2, LIG4, KLF4, NOTCH2, FOXA1), while others decreased after puberty (IGF1, KLF4, POSTN, NOTCH2, LIG4). Some of them have been investigated in BC. For example, DICER1 is thought to be involved in tumorigenesis. Its down-regulation may be related to the metastatic spread of tumors [46]. The IGF pathway has also been linked to mammary carcinogenesis in animal models. Higher IGF1 circulating levels significantly increased cancer risk, including premenopausal BC [47]. Other genes that were differentially expressed in our animals have uncertain relevance. For example, KLF4 may act as a transcriptional activator or repressor depending on the promoter context and/or cooperation with other transcription factors. There is no consensus on whether KLF4 functions as a tumor suppressor or an oncogene in BC [48–50]. Similarly, the role of STAT5A/B in BC is complex. In early stages of BC, STAT5A/B may promote malignant transformation and enhance growth of the tumor. This is in contrast to established BC, where STAT5A/B may mediate the critical cues for maintaining differentiation [51]. TGFB2 as part of the TGF-beta pathway is generally antiproliferative in epithelial cells but can also play either a tumor-suppressing or a tumor-promoting role in human breast carcinogenesis, depending on age and pathologic features of prognostic significance [52]. LIG4 encoding the protein DNA ligase IV that is involved in double-strand break repair has been shown either to have no significant relationship to BC risk [53, 54] or to be associated with a decrease in BC risk [55].

For other so-called BC stem cell markers, only sparse data are available so far. RIF1 contributes to ATM-mediated protection against DNA damage. There is some evidence that chromosome rearrangements such as RIF1 play an important role in BC development [56]. There are some links between BC and the transcriptional factor FOXA1, [57] NEK10, [58], and NOTCH2 [59].

The IGHG1 protein has been shown to be down-regulated in BC, [60] whereas ELF5 was expressed at higher levels in BC cells than normal epithelial cells [61]. GATA3 has been shown to drive invasive BC cells to undergo reversal of epithelial–mesenchymal transition, leading to the suppression of cancer metastasis [62]. To date, there are no data available on BC and BMPR1A, or LRRN3.

Extracellular matrix (ECM) molecules may provide important clues regarding the differentiation and biologic behavior of normal and neoplastic breast tissue. The high expression of POSTN seen in the adolescent breast in this study is interesting in light of the observation that POSTN overexpression in BC is associated with the development of bony metastases [63]. It may that the abnormal re-expression of this developmentally expressed protein contributes to the bone tropism of metastatic BC. Variations in other ECM molecules are less profound and may be explained at least in part by the relative proportions of stroma to glandular tissue in the tissues examined.

Diagnostic molecular tools have the potential to help clinicians decide how to treat a growing population of patients with early-stage BC. The MammaPrint<sup>®</sup> assay, developed in 2001 at the Netherlands Cancer Institute in Amsterdam, provides an expression profile of breast tumors for BC prognosis and prediction. The signature includes 70 relevant genes covering the hallmarks of cancer: cell cycle, metastasis, angiogenesis, and invasion [20]. In our data set, we found six genes covered by the MammaPrint<sup>®</sup> to be significantly regulated across life stages. GSTM3, Peci, TGFB3, IGFBP5, and SERF1A gene expression was reduced with advancing age, whereas CENPA gene expression increased until pregnancy followed by a significant decrease during lactation and postmenopause. Glutathione S-transferases such as GSTM3 metabolize a broad range of xenobiotics and carcinogens [64]. Peci is an auxiliary enzyme that catalyzes an isomerisation step required for the beta-

oxidation of unsaturated fatty acids [65]. Expression of SERF1A, IGFB5, and the centromeric protein CENPA [66] is down-regulated in tumor tissue [20]. The function of SERF1A has not been elucidated yet.

This is the first study to investigate gene expression profiles in normal breast tissue across life stages. Our data demonstrate distinct patterns of gene expression during breast development and may shed light on the profound effect of reproductive life history on BC risk.

There has not been performed a comparable study in humans so far. In the mouse model, comparative gene expression analysis has focused on pregnancy, lactation, and involution after weaning [67–69]. Broader assessments across the lifespan have not been reported. In the murine gland, PCA across pregnancy, lactation, and involution revealed three distinct gene expression profiles with the majority of genes being associated with the “proliferation” profile during late pregnancy, stable expression during lactation, and diminished expression during involution [68]. Gene ontology analysis showed that biological processes such as ion transport, calcium-mediated signaling, transferase activity, and cell proliferation were mostly represented by the dominant “proliferation” PCA [68]. When focusing on single gene groups, adipocyte specific genes (fat specific protein 27, resistin, adiponectin, CAP, perilipin, hormone stimulated lipase, mitochondrial, dicarboxylic amino acid transporter) were shown to decline throughout pregnancy and early lactation, while milk protein genes (e.g., various caseins, mucin 1, a-lactalbumin, butyrophilin, xanthine oxidoreductase, parathyroid hormone related protein) increased over the time period. These findings are in agreement with our data. Genes associated with the regulation of glucose entry and utilization for lactose synthesis were mostly up-regulated at parturition (e.g., glucose transporter 1, citrate synthase, citrate transporter, ATP citrate lyase). Similarly, many genes that determine the nature of the fatty acids in the triglyceride were up-regulated at secretory activation [67, 70]. In their model of secretory activation, Rudolph et al. suggested a positive regulatory role for prolactin and the PRLR signaling pathway via Akt1 (fatty acid synthesis, lactose synthesis) and STAT5 (protein synthesis), while progesterone was thought to act as a negative regulator via direct prolactin inhibition and indirectly by IGFBP5, TGFB2, and Wnt5b signaling [68]. Similarly, our data showed a constantly high expression of PRLR, STAT5, and ELF5 during pregnancy and lactation, while the expression of the negative regulator TGFB2 and IGFBP5 was lowest during lactation.

The study described here has its limitations. Since this is an exploratory study design, many questions regarding single gene function cannot be answered. Furthermore, as mentioned above, since adult animals in this study were obtained as adults from a breeding colony, they are likely multiparous. The tissues examined were composed of a variety of cell types, and the proportions of tissues varied across the lifespan. Thus, some effects, such as the relatively high expression of collagens in the juvenile stages, likely represent the preponderance of fibroblasts in the tissue sampled.

Most interestingly, a variety of genes including BC stem cell genes currently discussed as potential biomarkers are expressed already early in life. Thus, one might speculate that, firstly, some developmental markers presented here may also serve as biomarkers in BC; secondly, most genes expressed later in life when BC risk is higher have already been expressed during pubertal breast development suggesting a “thumb print” of life events in breast tissue, which, thirdly, leads to the hypothesis that exposure to agents early in life (hormones, environmental and dietary factors, etc.) may alter the gene expression profile of the breast, thereby altering BC risk later in life.

## Conclusion

Our data demonstrate distinct patterns of gene expression during breast development. Several of the pathways activated during pubertal development have been implicated in cancer development and metastasis, suggesting that developmental signals may have application as biomarkers for BC in later life. These data also illuminate imprinting effects in early life, as many genes associated with early breast differentiation persisted into later life. Possibly, therapeutic interventions early in life may alter BC risk later in life.

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## Abbreviations

|                               |  |
|-------------------------------|--|
| <b>AKR1C4</b>                 | Aldo-keto reductase family 1, member C4                    |
| <b>AKT1</b>                   | v-akt Murine thymoma viral oncogene homolog 1              |
| <b>ALDH1</b>                  | Aldehyde dehydrogenase 1 family, member A1                 |
| <b>AR</b>                     | Androgen receptor  |
| <b>BC</b>                     | Breast cancer  |
| <b>BMPRI1A</b>                | Bone morphogenetic protein receptor, type 1A               |
| <b>BRCA1</b>                  | Breast cancer 1  |
| <b>CAP</b>                    | Cyclase-associated protein                                 |
| <b>CD24, CD29, CD44, CD61</b> | Cluster designation antigens 24, 29, 44, and 61            |
| <b>CENPA</b>                  | Centromeric protein A                                      |
| <b>COL12A1</b>                | Collagen, type XII, alpha-1                                |
| <b>COL5A2</b>                 | Collagen, type V, alpha-2                                  |
| <b>CSN1S1</b>                 | Casein alpha S1  |
| <b>DICER1</b>                 | Dicer 1, ribonuclease type III                             |
| <b>ELF5</b>                   | E74-like factor 5  |
| <b>ER</b>                     | Estrogen receptor alpha (protein)                          |
| <b>ER</b>                     | Estrogen receptor beta (protein)                           |
| <b>ESR1</b>                   | Estrogen receptor alpha (gene)                             |
| <b>ESR2</b>                   | Estrogen receptor beta (gene)                              |
| <b>FOXA1</b>                  | Forkhead box A1  |
| <b>FOXC1</b>                  | Forkhead box C1  |
| <b>GATA3</b>                  | Glutamyl-tRNA amidotransferase subunit A binding protein 3 |
| <b>GH</b>                     | Growth hormone   |

|                |  |
|----------------|--|
| <b>GREB1</b>   | Gene regulated by estrogen in breast cancer 1      |
| <b>GTM3</b>    | Glutathione s-transferase mu 3                     |
| <b>HSD11B1</b> | 11-beta-hydroxysteroid dehydrogenase type 1        |
| <b>HSD17B2</b> | 17-beta-hydroxysteroid dehydrogenase type 2        |
| <b>HSD17B7</b> | 17-beta-hydroxysteroid dehydrogenase type 7        |
| <b>IGF1</b>    | Insulin-like growth factor 1                       |
| <b>IGFBP1</b>  | Insulin-like growth factor-binding protein 1       |
| <b>IGFBP5</b>  | Insulin-like growth factor-binding protein 5       |
| <b>IGHG1</b>   | IgG heavy-chain locus                              |
| <b>Jak2</b>    | Janus kinase 2                                     |
| <b>KLF4</b>    | Kruppel-like factor 4                              |
| <b>LAMC1</b>   | Laminin, gamma-1                                   |
| <b>LIG4</b>    | DNA ligase IV                                      |
| <b>LRRN3</b>   | Leucine-rich repeat protein, neuronal, 3           |
| <b>MKI67</b>   | Proliferation-related antigen Ki67                 |
| <b>NEK10</b>   | Never in mitosis gene a-related kinase 10          |
| <b>NOTCH2</b>  | Notch gene homolog 2                               |
| <b>PCA</b>     | Principal components analysis                      |
| <b>PDGFRA</b>  | Platelet-derived growth factor receptor, alpha     |
| <b>PECI</b>    | Peroxisomal D3, D2-enoyl-CoA isomerase             |
| <b>PGR</b>     | Progesterone receptor                              |
| <b>PGRB</b>    | Progesterone receptor B                            |
| <b>POSTN</b>   | Periostin  |
| <b>PPM1K</b>   | Protein phosphatase, PP2C domain-containing, 1K    |
| <b>PRLR</b>    | Prolactin receptor                                 |
| <b>RANKL</b>   | Receptor activator of NF-kappa-B ligand            |
| <b>RIF1</b>    | RAP1 interacting factor homolog                    |
| <b>SERF1A</b>  | Small EDRK-rich factor 1A                          |
| <b>STAT5</b>   | Signal transducer and activator of transcription 5 |
| <b>STS</b>     | Steroid sulfatase                                  |
| <b>SULT</b>    | Sulfotransferase                                   |
| <b>TFF1</b>    | Trefoil factor 1                                   |
| <b>TGFB1</b>   | Transforming growth factor, beta-1                 |
| <b>TGFB2</b>   | Transforming growth factor, beta-2                 |
| <b>TGFB3</b>   | Transforming growth factor, beta-3                 |
| <b>WNT5B</b>   | Wingless-related MMTV integration site 5B          |

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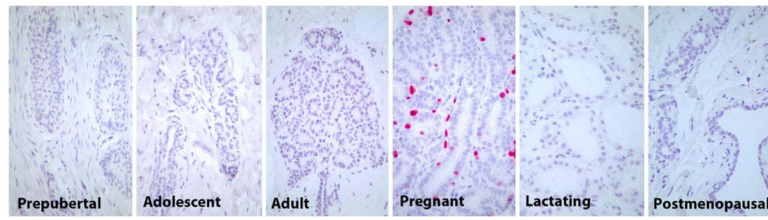
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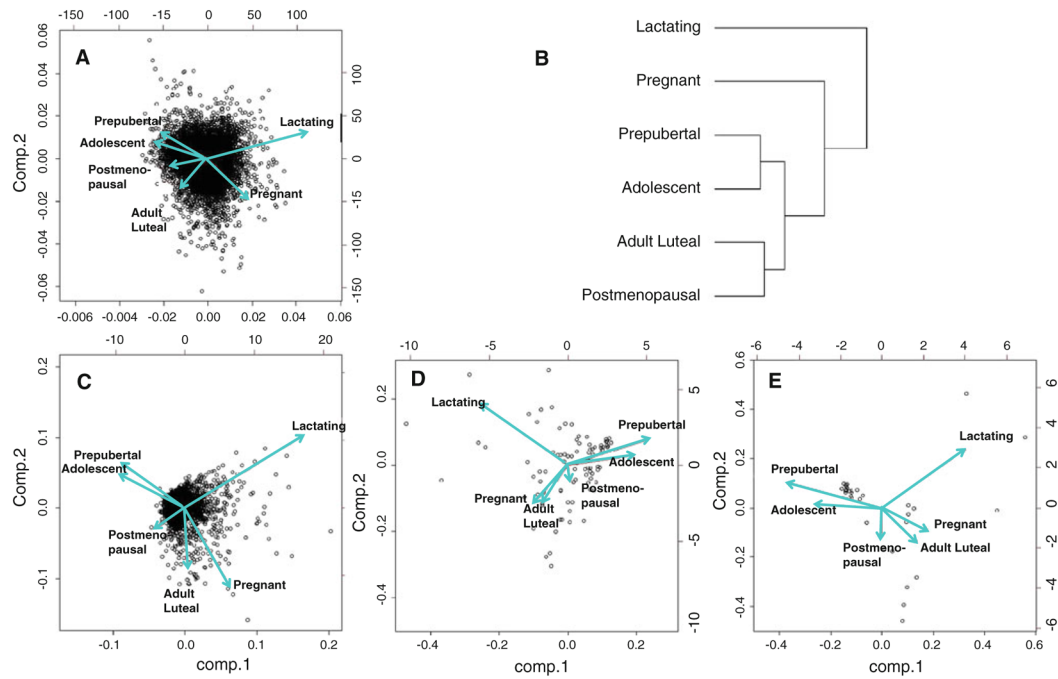


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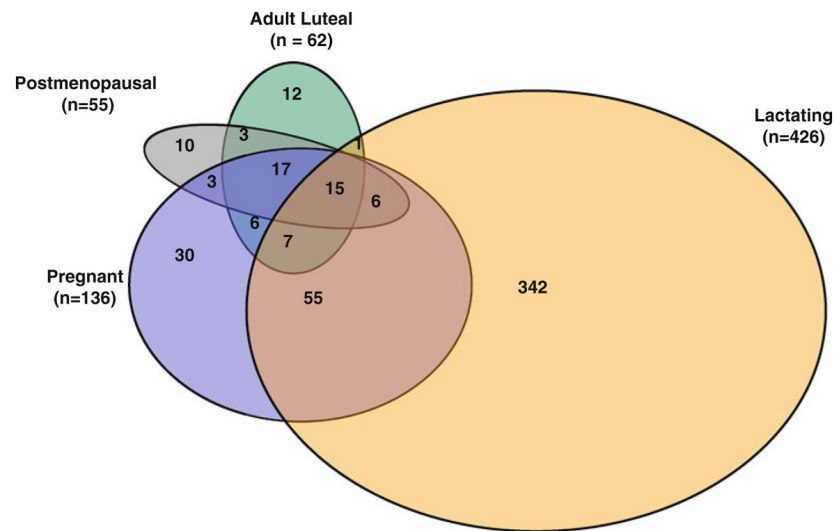
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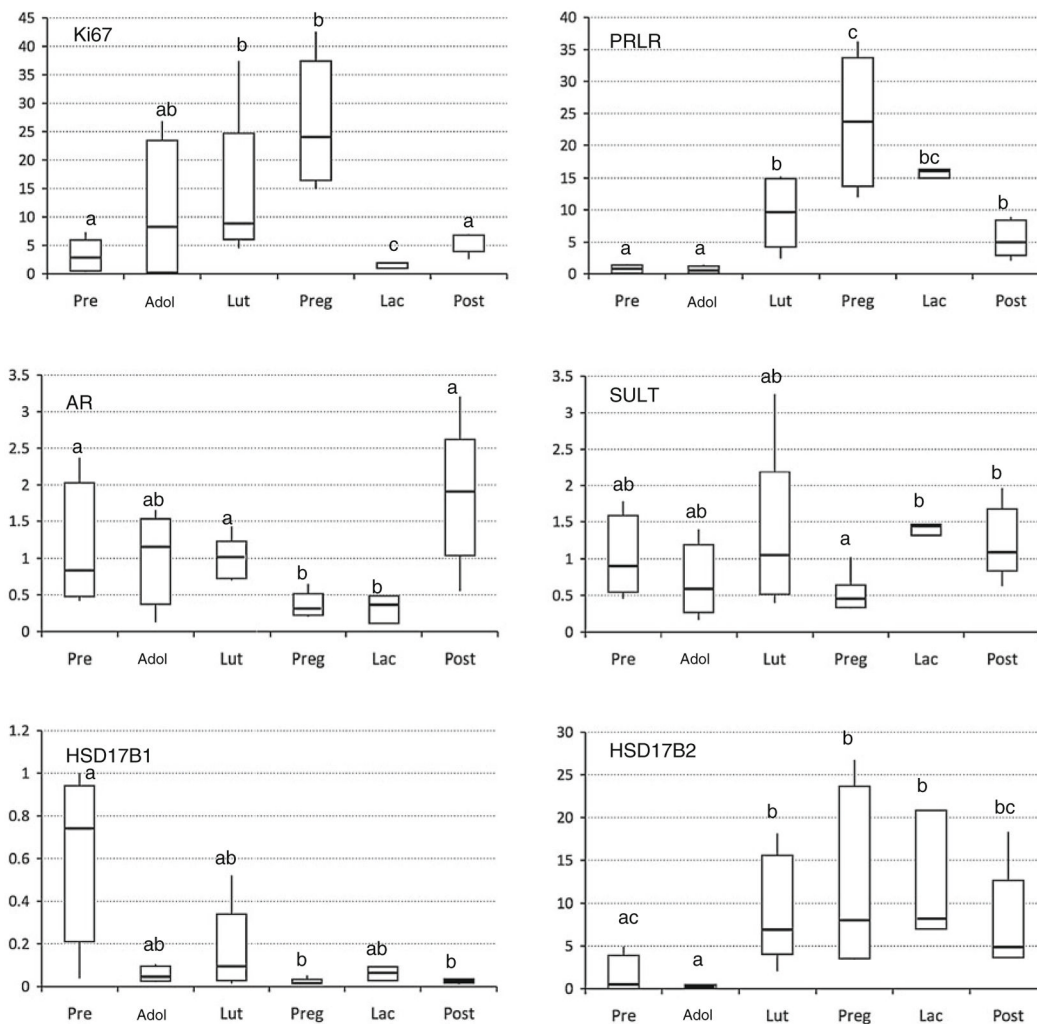
**Fig. 1.** Photomicrographs of breast histology for each life stage studied. Objective magnification was the same ( $\times 40$ ) for all images. Immunohistochemical stain for the proliferation marker Ki67; hematoxylin counterstain



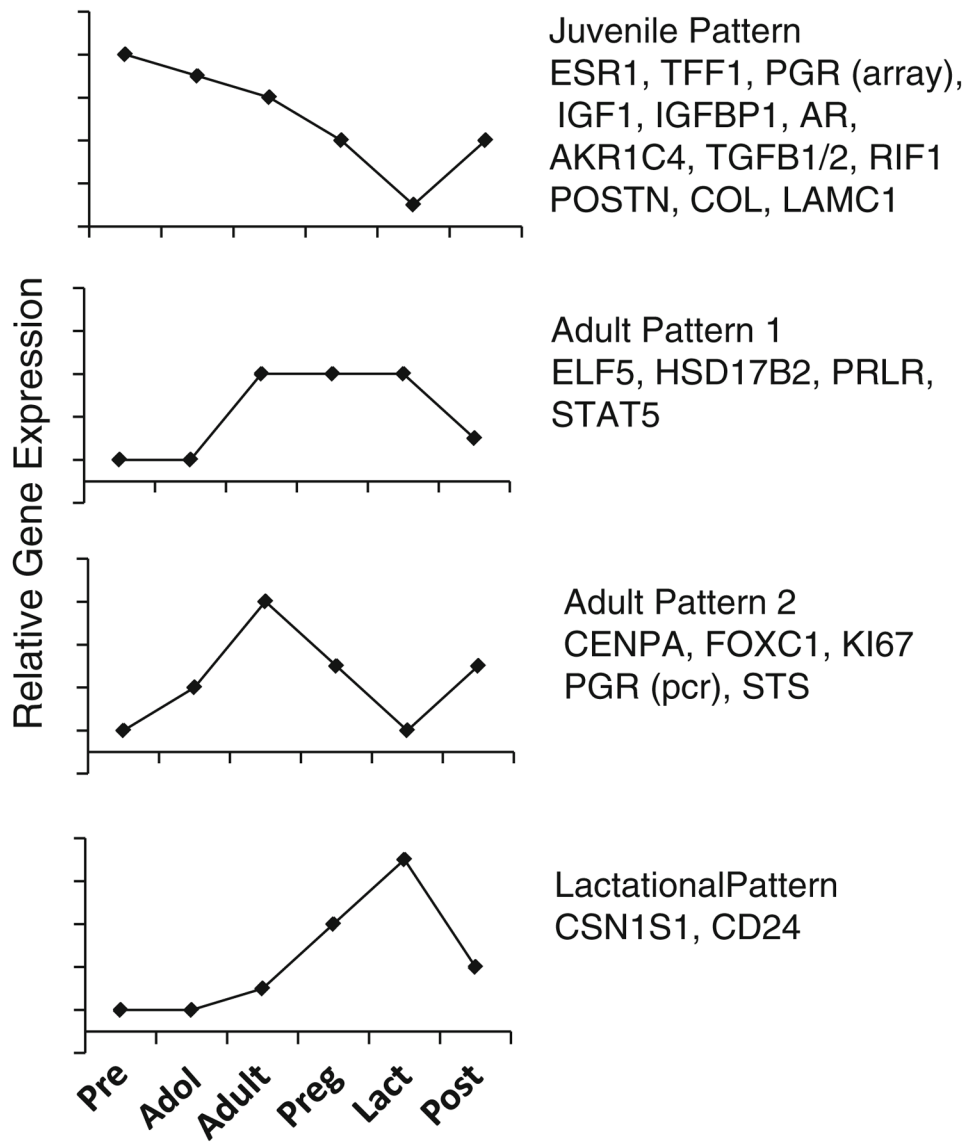
**Fig. 2.** Principal components analysis (PCA) of gene expression profiles in the breast across life stages and corresponding hierarchical clustering dendrogram (b). Vector diagrams correspond to the following gene filters: all genes detected (quality > 2,  $n = 26,484$ ) (a); genes significantly altered (ANOVA  $P_{adj} < 0.05$ , quality > 2) with FC > 1.2 ( $n = 1,964$ ) (c), 2.0 ( $n = 89$ ) (d), and 3.0 ( $n = 31$ ) (e) in all groups combined versus prepubertal



**Fig. 3.** Venn diagram presenting overlapping genes within life stage groups being significantly different from prepubertal animals at threshold fold change  $>10$  and  $P_{\text{adj}} < 0.05$ . There are no genes in the adolescent group falling into this category



**Fig. 4.** Gene expression measurements taken by qRT-PCR. *Box plots* of the 6 out of 13 genes examined with significant overall ANOVA at  $P < 0.05$ . Significant pairwise differences are indicated by differing symbols.  $P$  values for pairwise differences are  $<0.01$  (MKI67, PRLR, AR, and 17BHSD2),  $<0.03$  (17BHSD1), or  $<0.05$  (SULT)



**Fig. 5.** Schematic illustration of predominant patterns of gene expression from the microarray data. Y axis is not to scale

Table 1

Characteristics of animals in the study

| Group                | N | Age (year)       | Body weight (kg) | Estradiol (pg/ml)          | Progesterone (ng/ml)   |
|----------------------|---|------------------|------------------|----------------------------|------------------------|
| Prepubertal          | 4 | 2.8 <sup>a</sup> | 1.9 ± 0.1        | 12.3 ± 8.5                 | 1.1 ± 0.7              |
| Adolescent           | 4 | 2.8 <sup>b</sup> | 2.2 ± 0.3        | 4.6 ± 2.4                  | 0.9 ± 0.7              |
| Adult (luteal phase) | 5 | 13.2 ± 5.8       | 3.5 ± 0.5        | 23.2 ± 15.6                | 2.2 ± 0.7              |
| Pregnant             | 6 | 10.4 ± 2.8       | 3.3 ± 0.7        | 373.1 ± 151.3 <sup>*</sup> | 5.2 ± 0.6 <sup>*</sup> |
| Lactating            | 3 | 8.1 ± 1.8        | 3.8 ± 2.0        | 8.6 ± 0.6                  | 1.5 ± 1.0              |
| Postmenopausal       | 5 | 18.4 ± 3.7       | 3.5 ± 0.9        | 3.1 ± 0.0                  | 0.7 ± 0.7              |

Data are means ± standard deviation. Ages are estimated based on dentition

<sup>\*</sup> Different from all other groups at  $P < 0.05$  (Tukey's HSD test)<sup>a</sup> Sample collected greater than 6 months before menarche<sup>b</sup> Sample collected within 6 months after menarche



**Table 2**

Gene expression microarray data

|        | Prepubertal (a) | Adolescent (b) | Adult luteal (c) | Pregnant (d) | Lactating (e) | Postmenopausal (f) | Pairwise comparisons $P_{adj} < 0.05$   |
|--------|-----------------|----------------|------------------|--------------|---------------|--------------------|---|
| CSNIS1 | 1.00            | 2.40           | 35.54            | 43.76        | 101.24        | 22.66              | a vs. c-f<br>b vs. c-e<br>e vs. c, d    |
| ELF5   | 1.00            | 1.22           | 10.59            | 9.16         | 12.87         | 2.35               | a, b, f vs. c-e                         |
| GREB1  | 1.00            | -1.42          | -13.58           | -12.76       | -27.89        | -29.17             | a vs. c-f<br>b vs. c-e<br>d vs. e       |
| IGFBP1 | 1.00            | 1.34           | -89.23           | -86.31       | -83.32        | -19.67             | a vs. c-f<br>b vs. c-e                  |
| IGHG1  | 1.00            | 1.11           | 42.67            | 36.76        | 15.45         | 34.62              | a vs. c-f<br>b vs. c-e<br>c vs. e       |
| LRRN3  | 1.00            | -1.57          | -18.72           | -23.83       | -16.31        | -16.24             | a vs. c-f<br>b vs. c-e<br>d vs. f       |
| NEK10  | 1.00            | -1.43          | -14.73           | -30.99       | -14.18        | -10.09             | a vs. c-f<br>b vs. c-e<br>d vs. e       |
| PGR    | 1.00            | -1.02          | -8.95            | -23.77       | -112.26       | -15.81             | a vs. c-f<br>b vs. c-e<br>e vs. c, d, f |
| PPM1K  | 1.00            | -1.74          | -12.21           | -12.53       | -7.08         | -12.44             | a vs. c-f<br>b vs. c-e<br>d vs. e       |
| TFF1   | 1.00            | 1.16           | -84.59           | -168.88      | -97.09        | -57.84             | a vs. c-f<br>b vs. c-e                  |

Fold change in relative expression for adult life stage groups significantly different from prepubertal animals at threshold >10 and  $P_{adj} < 0.05$ . Prepubertal and adolescent groups do not differ from each other

**Table 3**

Gene expression array data for sex steroid receptors and metabolism

|         | Prepubertal (a) | Adolescent (b) | Adult luteal (c) | Pregnant (d) | Lactating (e) | Postmenopausal (f) | Pairwise comparisons $P_{adj} < 0.05$                      |
|---------|-----------------|----------------|------------------|--------------|---------------|--------------------|--|
| ESR1    | 1.00            | -1.34          | -5.01            | -4.79        | -3.36         | -5.10              | a vs. b-f<br>b vs. c-f<br>c vs. e                          |
| PRLR    | 1.00            | 1.26           | 3.20             | 4.51         | 1.88          | 1.61               | a vs. b-f<br>b vs. d<br>c vs. e<br>d vs. e, f              |
| PGRB    | 1.00            | -1.02          | -8.95            | -23.77       | -112.26       | -15.81             | a, b vs. c-f<br>e vs. c, d, f                              |
| AR      | 1.00            | 1.33           | -2.10            | -2.72        | -7.54         | -1.18              | a vs. b-f<br>b vs. c-e<br>c vs. e<br>d vs. e, f<br>e vs. f |
| MKI67   | 1.00            | 1.06           | 1.08             | 1.03         | 1.18          | -1.17              | a vs. b-f<br>c vs. d, e<br>d vs. e, f<br>e vs. f           |
| ST5     | 1.00            | -1.17          | -1.23            | -1.31        | -1.43         | 1.32               | a vs. b-f<br>b, c, f vs. d, e                              |
| HSD11B1 | 1.00            | 1.53           | -1.21            | -2.04        | -1.35         | -1.05              | a vs. b-f<br>d vs. b, c, f                                 |
| HSD17B7 | 1.00            | -1.12          | -1.21            | -1.84        | -1.53         | -1.10              | a vs. b-f<br>b, f vs. d, e<br>c vs. d                      |
| HSD17B2 | 1.00            | 1.03           | 4.66             | 4.92         | 3.11          | 3.31               | a vs. b-f<br>b vs. c-e                                     |
| AKR1C4  | 1.00            | -1.56          | -1.34            | -2.93        | -3.14         | -1.52              | a vs. b-f<br>b vs. d<br>c, f vs. d, e                      |

Up- and down-regulated overlapping genes within life stage groups significantly different from the prepubertal group at  $P < 0.05$ . Pairwise comparisons at  $P_{adj} < 0.05$  were made, and the relevant results are presented in the text

**Table 4**

Mammary progenitor cell markers

|        | Prepubertal (a) | Adolescent (b) | Adult luteal (c) | Pregnant (d) | Lactating (e) | Postmenopausal (f) | Pairwise comparisons $P_{adj} < 0.05$               |
|--------|-----------------|----------------|------------------|--------------|---------------|--------------------|---|
| ELF5   | 1.00            | 1.22           | 10.59            | 9.16         | 12.87         | 2.35               | a, b, f vs. c-e                                     |
| CD24   | 1.00            | 1.09           | 1.66             | 1.21         | 3.97          | 1.59               | a vs. b-f<br>e vs. b-d, f                           |
| STAT5  | 1.00            | 1.17           | 2.81             | 5.96         | 1.68          | 1.14               | a vs. b-f<br>b vs. d<br>c, f vs. d, e<br>d vs. e    |
| ALDH1  | 1.00            | 1.10           | 1.38             | -1.36        | 1.47          | 1.19               | a vs. b-f<br>b vs. d<br>d vs. e, f                  |
| DICER  | 1.00            | -1.23          | -1.25            | -2.25        | -4.02         | -1.20              | a vs. b-f<br>b, c vs. d, e<br>d vs. e, f<br>e vs. f |
| RIF1   | 1.00            | -1.37          | -1.29            | -2.44        | -4.49         | -1.44              | a vs. b-f<br>b, c vs. d, e<br>e vs. f               |
| BMPRIA | 1.00            | -1.12          | -1.14            | -1.56        | -1.56         | -1.16              | a vs. b-f<br>b vs. d<br>c, f vs. d, e               |
| TGFB2  | 1.00            | -1.40          | -1.17            | -3.99        | -6.23         | -1.22              | a vs. b-f<br>b, c, f vs. d, e                       |
| CD44   | 1.00            | -1.09          | -1.30            | -2.99        | -5.09         | -1.15              | a vs. b-f<br>b vs. d, e<br>c vs. e                  |
| CD29   | 1.00            | 1.00           | -1.24            | -1.85        | -4.86         | -3.86              | a vs. b-f<br>b, c, f vs. d, e                       |
| FOXA1  | 1.00            | -1.04          | -1.76            | -2.28        | -2.42         | -1.44              | a vs. b-f<br>b, c, f vs. d, e                       |
| KLF4   | 1.00            | 1.05           | 1.61             | -2.76        | -4.19         | 1.51               | a vs. b-f<br>b vs. c-e<br>c, f vs. d, e             |
| NOTCH2 | 1.00            | 1.17           | 1.19             | -1.58        | -2.37         | 1.11               | a vs. b-f<br>b, c vs. d, e<br>d vs. e, f<br>e vs. f |
| LIG4   | 1.00            | -1.12          | 1.37             | -2.03        | -4.09         | 1.12               | a vs. b-f<br>b vs. c-e<br>c vs. d, e<br>d vs. e, f  |

|       | Prepubertal (a) | Adolescent (b) | Adult luteal (c) | Pregnant (d) | Lactating (e) | Postmenopausal (f) | Pairwise comparisons $P_{adj} < 0.05$           |
|-------|-----------------|----------------|------------------|--------------|---------------|--------------------|---|
| BRCA1 | 1.00            | 1.11           | 1.06             | -1.18        | -1.68         | -1.22              | e vs. f<br>a vs. b-f<br>e vs. b-d, f            |
| POSTN | 1.00            | 1.16           | -10.06           | -28.27       | -92.92        | -9.17              | a vs. b-f<br>b vs. d-f<br>c, d vs. e<br>e vs. f |
| IGF1  | 1.00            | 1.28           | -2.46            | -2.22        | -8.22         | -2.28              | a vs. b-f<br>b vs. d-f<br>e vs. c, d<br>e vs. f |
| CD61  | 1.00            | -1.07          | 1.20             | -1.20        | 1.27          | -1.20              | a vs. b-f<br>e vs. b-d, f                       |

Up- and down-regulated overlapping genes within life stage groups being significantly different from prepubertal animals at  $P < 0.05$ . Pairwise comparisons at  $P_{adj} < 0.05$  were made, and the relevant results are presented in the text

**Table 5**

Extracellular matrix signaling

|         | Prepubertal (a) | Adolescent (b) | Adult luteal (c) | Pregnant (d) | Lactating (e) | Postmenopausal (f) | Pairwise comparisons $P_{adj} < 0.05$           |
|---------|-----------------|----------------|------------------|--------------|---------------|--------------------|---|
| POSTN   | 1.00            | 1.16           | -10.06           | -28.27       | -92.92        | -9.17              | a vs. b-f<br>b vs. d-f<br>c, d vs. e<br>e vs. f |
| TGFB1   | 1.00            | 1.06           | -2.46            | -4.90        | -14.89        | -1.30              | a vs. b-e<br>b vs. d, e<br>e vs. c, d, f        |
| COL5A2  | 1.00            | 1.15           | -3.78            | -4.27        | -20.15        | -2.68              | a vs. b-f<br>b vs. d, e<br>e vs. c, d, f        |
| COL12A1 | 1.00            | -1.05          | -2.49            | -5.53        | -10.87        | -1.77              | a vs. b-f<br>b vs. d, e<br>e vs. c, d, f        |
| FOXC1   | 1.00            | 2.07           | 9.15             | 4.49         | -1.74         | 2.89               | a vs. b-f<br>b, c vs. d, e<br>e vs. d, f        |
| LAMC1   | 1.00            | -1.08          | -1.61            | -3.77        | -8.55         | -1.26              | a vs. b-f<br>d vs. b, c<br>e vs. b-d            |
| PDGFRA  | 1.00            | -1.12          | -1.76            | -2.79        | -2.40         | -1.49              | a vs. b-f<br>b vs. d, e<br>c vs. d              |
| TGFB2   | 1.00            | -1.49          | -1.36            | -3.73        | -6.23         | -1.76              | a vs. b-f<br>b, c, f vs. d, e                   |

Up- and down-regulated overlapping genes within life stage groups being significantly different from prepubertal animals at  $P < 0.05$ . Pairwise Comparisons at  $P_{adj} < 0.05$  were made, and the relevant results are presented in the text

Table 6

Genes related to BC

|        | Prepubertal (a) | Adolescent (b) | Adult luteal (c) | Pregnant (d) | Lactating (e) | Postmenopausal (f) | Pairwise comparisons $P_{adj} < 0.05$                      |
|--------|-----------------|----------------|------------------|--------------|---------------|--------------------|--|
| SERF1A | 1.00            | -1.55          | -1.90            | -4.46        | -6.18         | -1.46              | a vs. b-f<br>b, c vs. d, e<br>e vs. f                      |
| GTM3   | 1.00            | -1.20          | -1.76            | -1.62        | -3.07         | -1.42              | a vs. b-f<br>e vs. b-d, f                                  |
| PECI   | 1.00            | -1.32          | -1.64            | -2.33        | -1.80         | -1.17              | a vs. b-f<br>b vs. d, e<br>e vs. f                         |
| IGFB5  | 1.00            | 1.47           | -1.15            | -2.72        | -8.67         | -1.69              | a vs. b-f<br>b vs. c-e<br>c vs. d, e<br>d vs. e<br>e vs. f |
| TGFB3  | 1.00            | 1.27           | -1.20            | -1.86        | -5.58         | -1.34              | a vs. b-f<br>e vs. b-d, f                                  |
| CENPA  | 1.00            | 1.45           | 2.08             | 1.63         | -2.16         | -1.45              | a vs. b-f<br>c, d vs. e, f<br>e vs. f                      |

Up- and down-regulated genes shared by adult life stage groups that were significantly different from prepubertal group at  $P_{adj} < 0.01$ . Pairwise comparisons at  $P_{adj} < 0.05$  were made, and the relevant results are presented in the text