Tumorigenesis and Neoplastic Progression

Alterations of Gene Expression in the Development of Early Hyperplastic Precursors of Breast Cancer

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Enlargement of normal terminal duct lobular units (TDLUs) by hyperplastic columnar epithelial cells is one of the most common abnormalities of growth in the adult female human breast. These hyperplastic enlarged lobular units (HELUs) are important clinically as the earliest histologically identifiable potential precursor of breast cancer. The causes of the hyperplasia are unknown but may include estrogen-stimulated growth mediated by estrogen receptor- α , which is highly elevated in HELUs and may be fundamental to their development. The present study used DNA microarray technology and RNA from microdissected pure epithelial cells to examine changes in gene expression and molecular pathways associated with the development of HELUs from TDLUs. The results suggest that HELUs evolve from TDLUs primarily by reactivation of pathways involved in embryonic development and suppression of terminal differentiation. Changes in ERBB genes were particularly prominent, including a uniform switch in ligands for the ERBB1 receptor (14-fold decrease in epidermal growth factor and 10-fold increase in amphiregulin, respectively) in HELUs compared with TDLUs. Epidermal growth factor regulates terminal differentiation in adult breast and amphiregulin is critical to normal embryonic breast development. Because HELUs are such early potential precursors of breast cancer, targeting some of these alterations may be especially promising strategies for breast cancer prevention. (*Am J Pathol* 2007, 171:252–262; DOI: 10.2353/ajpath.2007.061010)

one of the most common abnormalities in the adult female human breast.^{1,2} These hyperplastic enlarged lobular units (HELUs) are often multifocal, bilateral, and up to 100-fold larger (volume and numbers of cells) than the TDLUs they evolve from (Figure 1), representing a major alteration of growth.² The majority of HELUs are lined by one or two layers of crowded columnar epithelial cells with minimal nuclear atypia, but a substantial minority exhibit more diverse histological features, contributing to the complex terminology that has evolved to describe them.^{1,2} The HELUs evaluated in this study were the common generally single-layer type with minimal nuclear atypia (see Supplemental Figure S1 at <http://ajp.amjpathol.org>), which would be referred to as “columnar cell change” using other currently popular terminology.³ We prefer the term HELUs over others because it conveys our viewpoint that epithelial hyperplasia and the resulting enlargement of lobules are the most fundamental characteristics and that the cells may then differentiate or progress in diverse ways depending on additional events (eg, mutations) that follow.

Histological, epidemiological, and more recent genetic evidence suggest that HELUs are forerunners of many other abnormalities in the breast, including microcysts, usual ductal hyperplasia, and atypical ductal hyperplasia (ADH).^{2,4,5} ADH is a well-established potential precursor of breast cancer^{6–8} and, in this sense, HELUs can be viewed as the earliest histologically identifiable lesion with premalignant potential, as suggested at least a century ago.^{1,9,10}

Recent studies from our laboratory demonstrated that the epithelial cells lining HELUs show highly elevated expression of nuclear estrogen receptor- α (ER- α), significantly increased proliferation, and significantly de-

Supported by the National Institutes of Health (National Cancer Institute grant U01-CA84243) and AstraZeneca Pharmaceuticals.

Accepted for publication March 20, 2007.

Supplemental material for this article can be found on <http://ajp.amjpathol.org>.

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Enlargement of normal terminal duct lobular units (TDLUs) by hyperplastic columnar epithelial cells is

Figure 1. **A** and **B:** HELUs are much larger (up to 100-fold increased volume and number of cells) than the normal TDLUs they evolve from (H&E stain). This study used DNA microarray technology and RNA isolated from nearly pure (>95%) populations of epithelial cells isolated by laser capture microdissection to compare gene expression profiles between paired TDLUs (**C–E**) and HELUs (**F–H**). Original magnifications, $\times 40$ (**A**, **B**).

creased apoptosis compared with adjacent TDLUs.² Estrogen, by binding and activating ER- α , regulates proliferation and apoptosis in the normal breast,^{11–13} suggesting that elevated ER- α may be a fundamental defect contributing to the development of HELUs, although the underlying reasons for elevated ER- α are unknown, and other mechanisms are almost certainly involved.

This study used DNA microarray technology to learn more about changes in gene expression and molecular pathways associated with the development of HELUs from TDLUs. Because HELUs are thought to be such an early potential precursor of breast cancer, identifying alterations associated with their development may reveal new and especially effective strategies for breast cancer prevention.

Materials and Methods

Tissue Samples

Samples for the microarray studies were derived from eight formalin-fixed paraffin-embedded tissue (FFPET) biopsies containing paired normal TDLUs and well-developed HELUs from noncancerous adult female human breasts obtained within the past 3 years. Populations of nearly pure (>95%) epithelial cells were isolated from the TDLUs and HELUs in each biopsy (~25,000 cells per sample) by laser capture microdissection (Veritas; Arc-turus, Mountain View, CA) (Figure 1). Ten additional paired samples of TDLUs and HELUs from independent FFPET biopsies were prepared in a similar manner for validation studies. Five of these ten samples also con-

tained ADH arising within HELUs, which were also harvested by laser capture microdissection. All samples were anonymous and obtained with institutional review board approval (protocol H-10493).

Microarrays

Total RNA for the microarray experiments was purified from the samples using the Optimum kit (Ambion, Austin, TX), which is based on proteinase K digestion followed by phase separation in a proprietary microfilter cartridge and incubation with DNase to remove contaminating genomic DNA, yielding from 50 to 300 ng per sample. The purified total RNA was then linearly amplified for two rounds and converted to cDNA using the Paradise kit (Arcturus), which is based on an oligo-dT primer and T7 RNA polymerase. The cDNA was then *in vitro* transcribed to biotin-labeled cRNA with the IVT labeling kit (Affymetrix, Santa Clara, CA).

Gene expression was measured using U133-X3P Human GeneChip oligo-based microarrays (Affymetrix). These microarrays contain transcripts for an estimated 47,000 genes and 61,359 probe sets (many genes have multiple probe sets). A large majority of the probe sets were constructed to be within 300 bases of the 3' end of the transcripts to accommodate the relatively fragmented RNA that is typically obtained from FFPET samples. In brief, amplified biotinylated cRNA was further purified with the RNeasy mini kit (Qiagen, Frankfurt, Germany) and fragmented (20 μ g of cRNA) in 5 $\times$ fragmentation buffer (200 mmol/L Tris-acetate, 500 mmol/L KOAc, and 150 mmol/L MgOAc at pH 8.1) at a 4:1 volume ratio (cRNA/buffer) for 10 minutes to a range of 35 to 200 bases to optimize hybridization. Fragmented cRNA (20 μ g) was then mixed with hybridization controls (bioB, bioC, bioD, and cre) and hybridized to the microarrays for 16 hours. After hybridization, the microarrays were washed, stained, and scanned to generate quantitative digital files. Affymetrix CEL files are available online at Gene Expression Omnibus (accession number GSE7377; <http://www.ncbi.nlm.nih.gov/geo/>). Measuring global gene expression in FFPET samples using microarray technology is challenging but feasible and accurate.¹⁴ The quality of the original RNA was inferred from the 3'/5' ratios of an internal control gene (GAPDH) and the percent present calls of genes expressed above background in the microarray data, which fell well within satisfactory ranges for FFPET-derived samples as defined by the manufacturers of the kit used to amplify the RNA (Arcturus) and the microarray chip (Affymetrix), respectively (average for TDLUs = 15.9 and 24.5%; average for HELUs = 18.3 and 25.7%).

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

qRT-PCR was used to measure the expression of selected genes starting with total RNA template from the original samples to confirm the ability of the microarrays to accurately measure gene expression in FFPET sam-

ples and from the independent validation samples to confirm further the biological findings overall. Briefly, first strand cDNA was synthesized from 1 to 5 ng of total RNA using the SuperScript II kit (Invitrogen, Carlsbad, CA). The quantitative PCR reactions were then performed using dual-labeled (5'/6-FAM and 3'/Black Hole Quencher) probes and GeneAmp Fast PCR Master Mix (Applied Biosystems, Foster City, CA) in an ABI 7500 Fast Real Time PCR System instrument (Applied Biosystems) with an initial denaturing cycle at 95°C for 20 seconds followed by 45 alternating production cycles of 95°C for 3 seconds and 60°C for 30 seconds.

Six genes of interest were evaluated in the original samples, including *EGFR*, *EGF*, *AREG*, annexin A8 (*ANXA8*), prominin 1 (*PROM1*), and v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog (*KIT*). *EGF* and *AREG* were further evaluated in the independent validation samples. Human β 2-microglobulin (*B2M*) was used as the internal reference gene in all analyses. Sequences for the probes and primers (Table 1) were designed using Primer Express Software v2.0 (Applied Biosystems) and synthesized by a commercial vendor (Eurogentec, San Diego CA).

Data Analysis

dChip software (<http://www.dchip.org>) was used for estimating expression and class comparisons. Normalization of raw data were by the invariant set normalization method, and expression was estimated by the perfect match only model. Differentially expressed genes were identified using the *t*-test, and the empirical false discovery rate was estimated by permutation as previously described.¹⁵ More than 70% of the genes measured in this study showed SDs (log2 ratio) <1. With this degree of variability, power = 0.7 to detect significant differences in expression at more than or equal to twofold; $P < 0.05 = 0.7$; power = 0.7 to detect differences at more than or equal to fourfold; $P < 0.001 = 0.7$; and power = 0.9 at more than or equal to fourfold; $P < 0.05 = 0.9$.¹⁶

BRB Array Tools software (<http://linus.nci.nih.gov/BRB-ArrayTools.html>) was used for pathway comparisons using the functional class scoring analysis algorithm, which provides a list of potentially differentially activated pathways (as defined by KEGG and BioCarta) based on having a higher number of differentially expressed genes in the pathways than expected by chance, using LS (mean negative natural logarithm of the *P* values of the appropriate single gene univariate test) and KS (maximum difference between *i*/N and p_i , where p_i is the *i*th smallest *P* value of the univariate test) statistics as previously described.¹⁷ Probesets were filtered before class comparisons, eliminating genes expressed at or below background, to mitigate multiple comparison errors.

Gene Batch Finder and Gene Ontology (GO) Summary software (<http://cgap.nci.nih.gov>) were used to identify GO Terms that were significantly different between TDLUs and HELUs, inferred from a list of differentially

Table 1. Sequences for Primers and Probes of Genes Evaluated by qRT-PCR

Gene	Sequence
EGFR forward primer	5'-GCACATTTTGGGAAGTTGCA-3'
EGFR reverse primer	5'-CTGCTCAAAGGGACAATATTCTTG-3'
EGFR probe	5'-TCTTCAAACGTGAAGCATTACAGAAACGCA-3'
EGF forward primer	5'-GTGTGCTGGACGCCTGTCT-3'
EGF reverse primer	5'-CTTACGGAATAGTGGTGGTCATCTT-3'
EGF probe	5'-TTTGCCCTGACTCTACTCCACCCCTC-3'
AREG forward primer	5'-ACCTGGAAGCAGTAACATGCAA-3'
AREG reverse primer	5'-ACTGTCAATCATGCTGTGAGTTTC-3'
AREG probe	5'-CACACCGTTCACCGAAATATTCTTGCTGA-3'
ANX8 forward primer	5'-CAGGACTGGCCCTCCAAGA-3'
ANX8 reverse primer	5'-CATCTCATCAGTCCCACGAATC-3'
ANX8 probe	5'-CTCGCCTGCCGCATACAGATCCTG-3'
PROM1 forward primer	5'-CCAGCAACGAGTCCTTCTATAGA-3'
PROM1 reverse primer	5'-CCATTCCCTGTGCGTTGAA-3'
PROM1 probe	5'-CAATCACTGAGCACTCTATACCAAAGCGTCAA-3'
KIT forward primer	5'-TCTTTTCTTTCAACTTGCAATCAA-3'
KIT reverse primer	5'-AAAGTGTGCTCAGAAAGACAGGATT-3'
KIT probe	5'-TCCAGGATAGTGGGCACCCCACTG-3'
B2M forward primer	5'-ATAATTCTACTTTGAGTGCTGTCTCCAT-3'
B2M reverse primer	5'-TGCCAGCCCTCCTAGAGCTA-3'
B2M probe	5'-CTGTGGAGCAACCTGCTCAGATACATCAA-3'

expressed genes from a supervised comparison using dChip (see Results). Go Terms correspond to biological processes, cellular components, and molecular functions and pathways as defined by the Gene Ontology Consortium (<http://www.geneontology.org>).

Ingenuity Pathway Analysis software (Ingenuity System, Redwood City, CA) was also used to identify potentially important differences in biological mechanisms and pathways between TDLUs and HELUs, inferred from a list of differentially expressed genes from a supervised comparison using dChip (see Results). This program generates a list of theoretical networks based on algorithms assigning a score based on the number of differentially expressed genes in a dataset that are within annotated pathways and interact in various ways (eg, direct protein binding, activation, and so forth). The higher the score, the more genes and potential interactions there are in the pathways, inferring that they may be important. The networks are unique but not mutually exclusive. Graphical tools allow visualization of potential gene interactions within networks. Typically, the highest scoring networks are merged and visualized together.

Results

Unsupervised and Supervised Analyses of Gene Expression

Eight paired samples of RNA isolated from microdissected pure epithelial cells of TDLUs and HELUs were evaluated for global gene expression using Affymetrix U133-X3P Human GeneChip microarrays. An unsupervised hierarchical clustering of normalized results using dChip software and 6023 genes filtered from 61,359 total probe sets (filtering criteria: $\geq 50\%$ present calls; $0.5 < \text{SD}/\text{mean} < 1000$ variation across samples; absolute ex-

pression > 50 U) resulted in near-perfect separation of case-matched TDLUs and HELUs (Figure 2), indicating that there are prominent underlying differences in gene expression associated with, and probably involved in, the development of HELUs from TDLUs.

Heat map (left) and enlarged dendrogram (below) from an unsupervised hierarchical clustering using 6023 filtered genes.

Figure 2. An unsupervised hierarchical clustering using 6023 filtered genes resulted in near-perfect separation of paired normal TDLUs and HELUs. In a supervised comparison (more than or equal to twofold; $P < 0.05$), 825 genes showed significantly different levels of expression.

A. Top 10 of 56 GO Terms which significantly discriminate TDLUs and HELUs as defined by GGAP GO Summary.

GO Id	GO Term	Genes in List	Fisher Exact
GO:0005006	Epidermal growth factor receptor activity	4/798	0.00136
GO:0017017	MAP kinase phosphatase activity	4/798	0.00369
GO:0007264	Small GTPase mediated signal transduction	30/798	0.00395
GO:0007169	Transmembrane receptor PTK signaling	16/798	0.00456
GO:0043560	Insulin receptor substrate binding	3/798	0.00526
GO:0007264	Chemokine activity	8/798	0.00585
GO:0042379	Chemokine receptor binding	8/798	0.00722
GO:0001664	G-protein coupled receptor binding	9/798	0.00769
GO:0008610	Lipid biosynthesis	21/798	0.00915
GO:0001838	Embryonic epithelial tube formation	3/798	0.01310

B. Top 12 of 17 pathways which significantly discriminate TDLUs and HELUs as defined by BioCarta and KEGG using BRB Array Tools.

Pathway Id	Pathway Description	Genes in List	LS P-val	KS P-val
BioCarta: h_eea1	The role of FYVE-finger proteins in vesicle transport	18	0.00001	0.00490
BioCarta:h_ERBB3	NRG receptor degradation protein-1 controls ERBB3 receptor recycling	13	0.00001	0.00246
BioCarta: h_plateletApp	Platelet amyloid precursor protein pathway	11	0.00010	0.00280
BioCarta:h_cb1	CBL-mediated ligand-induce down regulation of EGF receptors	24	0.00014	0.00790
BioCarta:h_spry	Sprouty regulation of tyrosine kinase signals	31	0.00046	0.15868
BioCarta:h_egfr_smrte	Map kinase inactivation of SMRT co-repressor	21	0.00067	0.05840
BioCarta:h_cardiacegf	Role of EGF receptor transactivation by GPCRs in cardiac hypertrophy	24	0.00101	0.06179
BioCarta:h_mta3	Down regulation of MTA-3 in ER-negative breast tumors	28	0.00121	0.06687
Kegg:hsa00500	Starch and sucrose metabolism	24	0.00160	0.00115
Kegg:hsa00130	Ubiquinone biosynthesis	47	0.00328	0.00077
BioCarta:h_Efp	Estrogen-responsive protein Efp controls breast tumor growth	18	0.00335	0.15194
Kegg:hsa00600	Sphingoglycolipid metabolism	16	0.01455	0.00049

C. Pathways which significantly discriminate TDLUs and HELUs based on an analysis restricted to direct interactions of proteins within all ontologies as defined by Ingenuity.

Figure 3. Selected results from pathway analyses using CGAP GO Summary (A), BRB Array Tools (B), and Ingenuity software (C), highlighting the potential importance of pathways involving ERBB genes (red type or box) and estrogen/ER-regulated genes (blue type or box) in the development of HELUs from TDLUs.

A supervised comparison using dChip software identified 825 genes expressed at more than or equal to twofold different levels ($P \leq 0.05$) in HELUs compared with TDLUs (Figure 2 and Supplemental Table S1, which can be viewed at <http://ajp.amjpathol.org>), ranging from a 17.5-fold increase in epiregulin to a 14.8-fold decrease in odd Oz/ten-m homolog 2. The median false discovery rate for this comparison was 7%, and thus, the 825 genes identified far exceeded the 58 genes (0.07×825) expected by chance alone. ER- α expression at the RNA level was up-regulated 2.4-fold ($P = 0.007$) in HELUs, consistent with our previous study showing highly elevated expression at the protein level.² ER- β expression was similar in HELUs and TDLUs. Overall, more genes were relatively up-regulated (514 of $825 = 62\%$) than down-regulated (311 of $825 = 38\%$).

Pathway Analyses

Three independent analytical strategies were used to identify biological pathways that might be operating differently in TDLUs and HELUs, inferred from differences in expression of individual genes in the pathways (Figure 3).

The first strategy used the 825 gene list from the supervised comparison (more than or equal to twofold, $P \leq 0.05$) described above and the CGAP GO Summary function to identify 56 GO Terms that were significantly different between TDLUs and HELUs. Four of the top 10 most significant GO Terms involved overlapping pathways in which ERBB genes played a central role. Pathways regulating inflammation/immunity (chemokines, chemokine activity, G-protein chemokine receptors), lipid metabolism, and embryonic development were also prominent.

The second approach used BRB Array Tools pathway analysis software and a list of 26,709 genes filtered from 61,359 total probe sets (filtering criteria: $\geq 30\%$ present calls; $0.5 < \text{SD}/\text{mean} < 1000$ variation across samples; absolute expression > 100 U) to identify 17 BioCarta/KEGG-defined pathways that were significantly different between TDLUs and HELUs. Six of the top 12 involved overlapping pathways in which ERBB genes were central. Two of the top 12 involved estrogen/ER- α -regulated pathways. Lipid/carbohydrate metabolism was also prominent.

The third strategy used Ingenuity Pathways Analysis software and the 825 gene list from the supervised comparison (more than or equal to twofold, $P \leq 0.05$) described above to identify 43 hypothetical networks of genes that were significantly different between TDLUs and HELUs. The top two (highest scoring) networks were merged and used to create graphic visualizations of potential connections restricted to direct interactions of genes in the networks within all functional ontologies as defined by Ingenuity. This model also emphasized potentially critical roles for ERBB and estrogen/ER- α -regulated genes in the development of HELUs from TDLUs.

Figure 4. Hierarchical clustering and gene list from a supervised comparison (more than or equal to twofold; $P \leq 0.05$) between paired samples of normal TDLUs and HELUs of expressed genes in the ERBB pathway as defined by Affymetrix.

Analyses of ERBB Pathway and Estrogen/ER-Regulated Genes

Because previous analyses suggested that changes in the expression of ERBB pathway genes may be especially important in the development of HELUs, dChip software was used to conduct a supervised comparison (more than or equal to twofold, $P \leq 0.05$) restricted to the expression of genes ($n = 22$) on the U133-X3P microarray in the ERBB pathway as defined by Affymetrix (Figure 4). Surprising and interesting differences were observed in the expression of ligands for epidermal growth factor receptor (EGFR)/ERBB1 in HELUs relative to TDLUs, including prominent increases in epiregulin (17.5-fold) and amphiregulin (AREG) (10.3-fold) and a prominent decrease in epidermal growth factor (EGF) (14.3-fold). There was also a substantial decrease (3.5-fold) in insulin receptor (INSR). There were no significant changes in expression of the ERBB receptors, including EGFR/ERBB1.

Previous analyses also suggested that estrogen/ER- α -regulated genes may be important in the development of HELUs. An unsupervised hierarchical clustering was conducted using 385 genes filtered (filtering criteria: $\geq 20\%$ present calls; $0.5 < \text{SD}/\text{mean} < 1000$ variation across samples) from a total list of 584 estrogen/ER- α -regulated genes (Figure 5). The gene list (see Supplemental Table S2 at <http://ajp.amjpathol.org>) was derived from published microarray studies of ER- α -positive breast cancer cell lines stimulated by estrogen.^{18–27} There was near-perfect separation of TDLUs and HELUs, indicating that there are substantial underlying differences in estrogen-regulated pathways mediated by ER- α . A supervised comparison (more than or equal to

Figure 5. An unsupervised hierarchical clustering using 385 genes filtered from a total list of 584 estrogen-regulated genes resulted in near-perfect separation of paired normal TDLUs and HELUs. In a supervised comparison (more than or equal to twofold; $P \leq 0.05$), 52 genes were expressed at significantly different levels in TDLUs and HELUs, including AREG and EGF in a manner consistent with their known regulation by estrogen.

twofold, $P \leq 0.05$) identified 52 differentially expressed genes (Figure 5 and Supplemental Table S3, which can be seen at <http://ajp.amjpathol.org>), including increased AREG (10.3-fold) and decreased EGF (14.3-fold) in HELUs.

Confirmation and/or Validation by qRT-PCR

Selected interesting genes (*EGFR*, *EGF*, *AREG*, *ANXA8*, *PROM1*, and *KIT*) were evaluated by qRT-PCR using total RNA from the original eight paired samples of TDLUs and HELUs (Figure 6). The findings were very similar (direction and magnitude) to the original microarray results for each of these six genes, confirming their validity. Additional analyses were performed for *EGF* and *AREG* using total RNA isolated from 10 independent paired samples of TDLUs and HELUs. Again, the results were very similar, providing further validation that the switch in these two ligands for *EGFR* (ie, prominent decreased expression of *EGF* and increased expression of *AREG*) is an important biological characteristic of HELUs. Five of the samples also contained areas of ADH arising in the HELUs, and the results were nearly identical in these two compartments.

Discussion

HELUs are a very common and prominent alteration of growth in the adult female human breast. The idea that HELUs might be precursors of breast cancer was pro-

Figure 6. qRT-PCR was used to confirm the differential expression of selected interesting genes, including epidermal growth factor receptor (*EGFR*), *EGF*, *AREG*, annexin 8 (*ANXA8*), prominin 1 (*PROM1*), and v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog (*KIT*). Changes (direction and magnitude) in all six genes were confirmed using total RNA from the original eight paired samples. *AREG* and *EGF* were further evaluated by qRT-PCR using total RNA from 10 additional paired samples of TDLUs and HELUs, with the same results as the original eight samples. Five of these 10 breasts also contained foci of ADH arising within HELUs, and the same patterns of expression in HELUs were observed in ADH.

posed as far back as the early 1900s, if not earlier,^{1,9,10} and it is consistent with more recent studies showing that they are on a histological continuum with breast cancer,^{2,4,5} that they are risk factors for developing breast cancer,^{28–30} and that they may share genetic alterations (allelic imbalances) with breast cancer, especially when they occur in the same breasts.^{31,32}

The underlying causes of the hyperplasia leading to HELUs are unknown. Some evidence suggests that estrogen may be involved, including the observations that HELUs are more common in premenopausal compared with postmenopausal breasts⁵ and in cancerous compared with noncancerous breasts^{5,30} where increased estrogen exposure is such a strong risk factor for developing cancer.³³ Recent studies from our laboratory demonstrated that the epithelial cells lining HELUs show highly elevated expression of ER- α , significantly increased proliferation, and significantly decreased apoptosis compared with TDLUs.² Because estrogen, mediated by ER- α , stimulates proliferation¹² and suppresses apoptosis¹³ in normal cells, elevated ER- α in HELUs may be a fundamental alteration leading to increased growth. A recent study in mice overexpressing ER- α in normal breast epithelium noted the rapid development of hyperplasia that occasionally progressed to cancer,³⁴ supporting the idea that elevated ER- α may be partially responsible for the development of HELUs and their progression to more advanced lesions in humans.

Because HELUs are so common in the population, relatively similar to each other in histological appearance (especially the frequent single-layer type evaluated in this study), and share important biological characteristics (eg, elevated ER- α), their beginnings seem more likely to reflect alterations in development or differentiation than genetic defects per se. Regardless, the end result is increased growth, creating fertile soil for accumulating random genetic defects leading to diversity and progression to more committed breast cancer precursors such as ADH.

This study used DNA microarray technology to learn more about changes in gene expression and molecular pathways associated with the development of HELUs from TDLUs. Several analyses identified pathways involving ERBB genes as important. A particularly interesting finding involved a switch in ligands for ERBB1/EGFR, including a uniform down-regulation of EGF (14-fold) and transforming growth factor- α (twofold) and up-regulation of AREG (10-fold) and epiregulin (17-fold) in HELUs compared with TDLUs. The changes in *EGF* and *AREG* were especially large in both absolute and relative terms. In contrast, expression of all ERBB receptors (including ERBB1/EGFR) did not change significantly. Recent studies have shown that estrogen, mediated by ER- α , can induce *AREG*^{35,36} and suppress *EGF*²¹ in normal and malignant breast epithelial cells. Thus, estrogen may be partially responsible for the switch in these ligands, particularly in the setting of the highly elevated ER- α observed in HELUs.²

There is ample evidence in breast and other types of cells that activation of ERBB1/EGFR by different ligands may have different functional consequences. For example, *AREG* is essential for ductal morphogenesis in the embryonic mouse mammary gland, whereas *EGF* is important for alveolar development in the adult.^{37,38} In monolayer cultures of human kidney Madin-Darby canine kidney cells expressing EGFR, AREG induces an epithelial-to-mesenchymal transformation, whereas EGF has no apparent effect.³⁹ In three-dimensional collagen gels, *EGF* also induces Madin-Darby canine kidney cells to form well-organized gland-like spheres and ducts, whereas AREG results in the formation of disorganized rudimentary structures,³⁹ suggesting that AREG disrupts differentiation in this setting. Collectively, this evidence suggests that the development of HELUs from TDLUs may involve reactivation of embryonic developmental pathways and/or inhibition of terminal differentiation partially mediated by up-regulation of *AREG* and down-regulation of *EGF*.

AREG has also been shown to enhance the tumorigenicity of certain human breast cancer cell lines⁴⁰ and to stimulate proliferation in the human premalignant cell line MCF10A.⁴¹ Baseline expression of AREG is very low in MCF10A but increases dramatically when the cells are transfected with *ras* and *c-neu* oncogenes, which also enhances their ability to progress to cancer in xenografts.⁴¹ Thus, AREG may help promote tumor progression, consistent with our observation that the up-regulation of AREG observed in HELUs was also present in ADH that evolved from the HELUs. These observations

suggest that pharmacological targeting of AREG may be an effective strategy for breast cancer prevention in high-risk patients with ADH.

Alterations of genes typically associated with inflammation and immunity may also be important in the development of HELUs. Several chemokines were uniformly down-regulated, including chemokine (C-X-C motif) ligand 5 (*CXCL5*, 7-fold), *CXCL2* (6.0-fold), *CXCL3* (5.8-fold), *CXCL12* (5.1-fold), chemokine (C-C motif) ligand 28 (*CCL28*, 4.5-fold), *CCL8* (3.4-fold), chemokine (C-X3-C motif) ligand 1 (*CX3CL1*, 3.0-fold), and *CXCL14* (2.1-fold). Most are potent attractants and activators of inflammatory cells, including neutrophils (*CXCL5*, *CXCL12*, and *CXCL14*), monocytes (*CCL8* and *CXCL14*), and lymphocytes (*CCL28* and *CCL8*).⁴² *CXCL14* has also been implicated in the differentiation of monocytes to macrophages.⁴³ The change in *CXCL12* was particularly large in both absolute and relative levels of expression. ER- α was recently shown to activate the transcription of *CXCL12* in breast cancer cell lines, resulting in increased proliferation,⁴⁴ but it seems unlikely to be stimulating growth in HELUs because it was down-regulated in this setting. *CXCL12* was also recently shown to be highly expressed in the myoepithelial cells of noninvasive breast cancer compared with normal TDLUs,⁴⁵ but our assessment of HELUs was restricted to epithelial cells, preventing comparison.

Several other genes usually associated with inflammation and immunity were also down-regulated in HELUs, including monocyte to macrophage differentiation-associated gene (*MMD*, 2.2-fold) and the transcription factors nuclear factor of κ light polypeptide gene enhancer in B-cells 1 (*NFKB1*, 2.1-fold) and nuclear factor κ B inhibitor ζ (*NFKBIZ*, 3.7-fold). The latter two also suppress apoptosis in certain cells,^{46,47} although they are unlikely to be involved in the suppression of apoptosis observed in HELUs² because both were down-regulated. Killer cell lectin-like receptor subfamily K member 1 (*KLRK1*) and T-cell receptor α constant (*TRAC*) were also decreased in HELUs (3.2- and 3.8-fold, respectively), which is surprising because the former is a receptor on natural killer cells mediating cytotoxicity,⁴⁸ and the latter is a component of the T-cell receptor.⁴⁹

It is possible that these and other genes mentioned above were measured in inflammatory cells contaminating the samples in our study. However, we think this is unlikely to be a major factor because the samples showed no obvious inflammation histologically, they were carefully microdissected to be composed of nearly pure populations of epithelial cells, and the expression of many of the genes in question was too high to be easily explained by contamination (eg, *NFKBIZ* and *TRAC*). Perhaps some of these genes are expressed in breast epithelium as well, which would be interesting. The cell source, however, is not important in the sense that they were still expressed at significantly reduced levels in HELUs compared with normal TDLUs.

Inflammatory cells play critical roles in the normal breast. For example, macrophages are essential during the involution of lactating breast tissue by inducing apoptosis of epithelial cells and participating in their phago-

cytic clearance.^{50,51} Normal luminal epithelial cells also participate in this process as cannibalistic phagocytes,⁵² and perhaps they express inflammation-associated genes to accomplish this. Similar processes may be involved in the remodeling of TDLUs during the menstrual cycle, although this has not been formally studied to our knowledge. Macrophages are also critical for development and differentiation in the embryonic mammary gland.⁵³ Our results suggest that suppression of inflammation and immunity may be important in the development of HELUs, perhaps by reducing cell turnover and/or inhibiting terminal differentiation during normal tissue remodeling.

Many other genes involved in development and differentiation also showed altered expression in HELUs. For example, several epithelial-specific Ets transcription factors⁵⁴ were uniformly down-regulated, including E74-like factor 5 (*ELF5*, 10.6-fold), Ets homologous factor (*EHF*, 5.3-fold), E74-like factor 3 (*ELF3*, 3.3-fold), and Ets variant gene 6 (*ETV6*, also known as the TEL oncogene; 3.1-fold). Other developmentally important transcription factors showing decreased expression included sex determining region Y box 9 (*SOX9*, 5.4-fold), *SOX8* (4.4-fold), *SOX10* (2.9-fold), mastermind-like 2 (*MAML2*, 3.6-fold), and forkhead box C1 (*FOXC1*, 10.1-fold).^{55–59} *PROM1* (CD133), *KIT*, and *ANXA8* were also significantly down-regulated in HELUs (4.2-, 3.1-, and 5.2-fold, respectively). *PROM1* and *KIT* are expressed in several types of stem cells,^{60,61} *KIT* may play a role in maintaining normal differentiation in the adult breast,⁶² and *ANXA8* is important during ductal morphogenesis in the embryonic breast.⁶³ *ANXA8* was also recently reported as being elevated in the myo-epithelial cells of ADH (which evolve from HELUs),⁶⁴ but our study was restricted to epithelial cells, barring comparisons. Transforming growth factor- β 1 suppresses proliferation in ER- α -positive/proliferating breast epithelium, which may represent an important progenitor cell population.⁶⁵ We recently reported a sixfold increase in ER- α -positive/proliferating cells in HELUs compared with TDLUs.² However, there were no changes in the expression of relevant transforming growth factor- β pathway genes at the RNA level in this study (data not shown), but it would be necessary to assess protein at the individual cellular level to exclude their involvement completely. Collectively, these data also suggest that delays in development and suppression of terminal differentiation are important in the evolution of HELUs from TDLUs.

In summary, HELUs are a common abnormality of growth in the breast and the first histologically identifiable potential precursor of breast cancer. To the extent that function can be inferred from static measurements of gene expression, our data suggest that the evolution of HELUs from TDLUs involves alterations leading to reactivation of embryonic development and/or suppression of terminal differentiation. Alterations of pathways involving ERBB genes were especially prominent, including a switch from adult to embryonic ligands for EGFR/ERBB1 (*EGF* and *AREG*, respectively). Suppression of many genes usually as-

sociated with inflammation and immunity also seem to be important, perhaps by delaying cell turnover during normal tissue remodeling. Finally, several of the most interesting changes involve estrogen-regulated genes, and the effects of these changes may be greatly augmented by the highly elevated expression of ER- α observed in HELUs. Elevated expression of ER- α may be a fundamental defect responsible for widespread hyperplasia leading to HELUs, setting the stage for further progression to more committed precursors of breast cancer.

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