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Cell Identity Disruption in Breast Cancer Precursors

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Abstract. *Background: Mammary epithelial cell identity depends on a set of genes epigenetically-regulated by maintenance proteins, the best-characterized of which belong to the Trithorax and Polycomb groups. Perturbations in expression of these proteins may disrupt cell identity and trigger tumor initiation. Materials and Methods: The pattern of expression of a panel of genes involved in control of cell identity and mammary gland remodeling was investigated in two pre-cancerous lesions, atypical ductal hyperplasia (ADH) and ductal carcinoma in situ (DCIS) and compared to the corresponding histologically normal tissue. Results: ADH and DCIS showed a close association in overexpression of Polycomb complex components, silencing of Homeobox A (HOXA) cluster gene, and overexpression of the genes involved in estrogen signaling, specifically, forkhead box A1 (FOXA1) and GATA binding protein 3 (GATA3) pioneer factors, and estrogen receptor-1 (ESR1). Conclusion: Our findings support the hypothesis that disruption of epigenetic control is associated with loss of cell identity and acquisition of a constitutive estrogen-dependent terminally-differentiated luminal phenotype.*

The terminal ductal lobulo-alveolar unit (TDLU) is the site from which most epithelial hyperplasias and carcinomas of the breast arise (1). Histologically, TDLU is composed of a bilayered and polarized epithelium: an inner sheet, termed luminal epithelium, and an outer sheet, termed basal epithelium or myoepithelium. Luminal and myoepithelial cells can be differentiated using cell type-specific markers (2).

Differently from many other organs, the developmental program of the mammary gland is completed only post-natally when, in response to ovarian hormones (estrogen and progesterone), extensive TDLU proliferation occurs: estrogen being responsible for ductal extension and progesterone for lobular differentiation.

According to the classical genomic model, ovarian hormones, specifically estradiol, bind to specific ligand-activated nuclear receptors, which after recognizing definite sequences within the promoter/enhancer region of a target gene, directly regulate its expression. Alternatively, according to the non-classical genomic model, the ligand – receptor complex can indirectly activate target gene transcription by physical interaction with other transcription factors (3). In both cases, besides ligand availability, estrogen receptor (ER) transcriptional activity depends on the cooperation of a class of proteins, collectively called co-regulators, that act as co-activators and co-repressors (4). As part of multicomponent protein complexes, co-regulators carry-out a wide range of enzymatic activities (acetylation, methylation and ubiquitination) that are required for correct gene transcription. In addition to co-regulators, other proteins, termed pioneer factors, are required for ER complex interaction with DNA. They belong to a special class of proteins able to associate directly with condensed chromatin independently of and prior to any other transcription factor, allowing accessibility to chromatin (5). In mammary gland, for example, forkhead box A1 (FOXA1) has proved to be a pioneer factor and act as master regulator of ER activity. Indeed, because of its ability to bind DNA and core histones simultaneously, FOXA1 disrupts their interactions meanwhile recruiting ER complex to the promoter of the target gene (6). Recently, GATA3, a transcription factor involved in mammary-gland morphogenesis and luminal-cell differentiation (7), was also proven to function as pioneer factor and acts *via* a cross-positive feedback loop in which expression of ER is required for the transcription of *GATA3* gene, and *GATA3* is required for estrogen receptor 1 (*ESR1*) transcription (8).

In normal TDLUs, however, estrogen stimulation affects only a small number of cells, since only about 30% of normal luminal cells express ER (9). In addition, these cells almost never divide and act on adjacent ER-negative cells by inducing their proliferation *via* paracrine factors produced in response to

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estrogen stimulus (10). Conversely, most primary breast carcinomas express high levels of ER in nearly all cells, the majority of which are dividing cells. Disruption of the mechanism governing the dissociation between steroid receptor expression and cell proliferation supports the emerging role of the ER pathway as autocrine signaling (11). According to the model for breast cancer development proposed by Dontu *et al*. (12), these dividing ER-positive cells should derive from transiently-amplifying ER-positive progenitor cells in which perturbation in the mechanisms appointed to control cell identity has occurred. Indeed, hormone-driven mammary gland remodeling is characterized by the acquisition of a specific and heritable cell identity depending on the differential expression of a predetermined set of genes under the epigenetic control of specific proteins termed maintenance proteins (13). This epigenetic control is achieved through DNA methylation, histone modification and chromatin remodeling (14). The best-characterized maintenance proteins belong to the Trithorax and Polycomb groups (15). Organized in multi-factor complexes, Trithorax and Polycomb proteins control the transcription of many genes that contribute to stem cell renewal, pluripotency and embryonic development (16, 17). Consequently, dysregulation in expression and activity of Trithorax and Polycomb group components may have dramatic effects on cell identity and trigger pathological transformation that may result in cancer (18).

The aims of the present study were to investigate in pre-cancerous lesions, specifically in atypical ductal hyperplasia (ADH) and ductal carcinoma *in situ* (DCIS), the pattern of expression of a panel of genes involved in the control of cell identity and mammary gland remodeling, in order to compare it against the corresponding patient-matched histologically-normal (HN) tissue, as well as to explore the relationship with the ER signaling pathway.

Materials and Methods

Tissue samples. We make use of a publicly accessible microarray dataset. As reported in the original article (19), patient-matched samples (HN, ADH, and DCIS) were isolated *via* laser capture microdissection from surgical specimens of 12 preoperative untreated patients with ER-positive (immunohistochemically- evaluated) sporadic breast cancer. Gene expression was determined by using the Affymetrix Human Genome HG-U133A GeneChip (Affimetrix, Santa Clara, CA, USA); the corresponding microarray dataset was publicly available at the ArrayExpress web site (http://ebi.ac.uk/arrayexpress/) with accession number E-GEOD-16873.

Gene set selection. To address the relation among genes involved in maintaining control of cell identity and mammary gland remodeling, and ER and progesterone receptor signaling, a panel of 129 genes was established (Table I). The genes were selected according to an extensive literature review. Twelve genes had no corresponding probe-sets on the HG-U133A GeneChip. Therefore, the gene set was actually composed of 117 elements: eight involved in nuclear receptor signaling, 44 in epigenetic control of gene transcription, 42 in development and cell fate-decisions, and 6 in cell growth control; 17 were used as epithelial luminal and basal markers. These 117 genes corresponded to 233 Affymetrix probe-sets, as verified by GeneAnnot system v2.2 (http://bioinfo2.weizmann.ac.il/geneannot/), which additionally provided us information about the quality of each probe-set in terms of sensitivity and specificity score (20).

Statistical analysis. As some genes are recognized by more than a single probe-set, each of which is characterized by an individual specificity and sensitivity that differently contributes to the gene expression value, prior to analysis, a mean gene expression value was calculated after weighting each probe-set for its own sensitivity and specificity score. Specifically, each expression value was multiplied by the semi-sum of sensitivity and specificity scores of the corresponding probe-set. Given the patient-matched samples of the study design, all statistical analyses were performed considering an ANOVA model for repeated measures with random effect. The differential gene expression among ADH, DCIS and HN was evaluated using the F-test after correction for multiple testing. To correct for multiple testing, the false discovery rate (FDR) was used (21). An FDR cut-off of 10% was used. The uncorrected *p*-value for the comparisons ADH *versus* HN and DCIS *versus* HN was finally reported. All analyses were performed using the open source software R 2.11.1 packages HDMD (http://www.R-project.org).

Results and Discussion

Out of the 117 genes considered in the study, 22 were found to be differentially expressed (global FDR<0.1) (Table II). Specifically, these genes were differentially expressed between DCIS and HN; 17 were also differentially expressed in ADH, suggesting their dysregulation as an early event in pathological transformation.

The set of genes differentially expressed (Figure 1) includes five genes coding for luminal markers [CD24 molecule (*CD24*), epithelial cell adhesion molecule (*EPCAM*), keratin 18 (*KRT18*), *KRT19*, and mucin 1 (*MUC1*)]; three for basal markers [actin alpha-2 (*ACTA2*), epidermal growth factor receptor (*EGFR*), and membrane metallo-endopeptidase (*MME*)]; three for nuclear hormone receptors (*ESR1*) and pioneer factors (*FOXA1* and *GATA3*); four for Trithorax [actin-like 6A (*ACTL6A*), and SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily e, member 1 (*SMARCE1*)] and Polycomb [chromobox homolog 8 (*CBX8*) and enhancer of zeste homolog 2 (*EZH2*)] proteins, and seven involved in cell fate [sex determining region Y-box 4 (*SOX4*)] and development [homeobox A1 (*HOXA1*), *HOXA2*, *HOXA4*, *HOXA5*, *HOXA7*, *HOXA9*].

Notably, compared to HN, ADH and DCIS had very similar gene expression profiles (Figure 2). Overexpression of genes coding for luminal markers was paralleled by underexpression of genes coding for basal markers, indicating that, with respect to HN tissue, both breast cancer precursors are characterized by a terminally-differentiated luminal

Table I. *Genes selected for the study (indicate with * genes without a corresponding HG-U133A probe-set).*

Group	Gene symbol	Gene name	Ensembl genomic location
Luminal markers	*CD24*	CD24 molecule	6q21
	CDH1	Cadherin 1, type 1, E-cadherin (epithelial)	16q22.1
	EPCAM	Epithelial cell adhesion molecule	2p21
	KRT7	Keratin 7	12q13.13
	KRT8	Keratin 8	12q13.13
	KRT18	Keratin 18	12q13.13
	KRT19	Keratin 19	17q21.2
	MUC1	mucin 1, cell surface associated	1q22
Basal markers	*ACTA2*	Actin, alpha 2, smooth muscle	10q23.31
	EGFR	Epidermal growth factor receptor	7p11.2
	KRT5	Keratin 5	12q13.13
	KRT6A	Keratin 6A	12q13.13
	KRT6B	Keratin 6B	12q13.13
	KRT14	Keratin 14	17q21.2
	KRT17	Keratin 17	17q21.2
	MME	Membrane metallo-endopeptidase (CD10)	3q25.2
	VIM	Vimentin	10p13
Nuclear receptors & co-regulators	*ESR1*	Estrogen receptor 1 (ERα)	6q25.1
	ESR2	Estrogen receptor 2 (ERβ)	14q23.2
	NCOA3	Nuclear receptor coactivator (AIB1/SRC3)	20q13.12
	NCOR1	Nuclear receptor corepressor 1 (N-CoR)	17p11.2
	NCOR2	Nuclear receptor corepressor 2 (SMRT)	12q24.31
	PGR	Progesterone receptor	11q22.1
Pioneer factors	*FOXA1*	Forkhead box A1	14q21.1
	GATA3	GATA binding protein 3	10p14
Trithorax proteins ATP-dependent chromatin-remodeling complexes	*ACTL6A*	Actin-like 6A (BAF53A)	3q26.33
	ACTL6B	Actin-like 6B (BAF53B)	7q22.1
	ARID1A	AT rich interactive domain 1A (SWI-like) (BAF250A)	1p36.11
	*ARID1B**	AT rich interactive domain 1B (SWI-like) BAF250B)	6q25.3
	*ARID2**	AT rich interactive domain 2 (ARID, RFX-like) (BAF200)	12q12
	BPTF	Bromodomain PHD finger transcription factor (NURF301)	17q24.2
	BRD7	Bromodomain containing 7	16q12.1
	CARM1	Coactivator-associated arginine methyltransferase 1	19p13.2
	PBRM1	Polybromo 1 (BAF180)	3p21.1
	RBBP4	Retinoblastoma binding protein 4 (RbAp48)	1p35.1
	RBBP7	Retinoblastoma binding protein 7 (RbAp46)	Xp22.2
	SMARCA1	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 1 (SNF2L)	Xq25
	SMARCA2	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 2 (BRM)	9p24.3
	SMARCA4	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 4 (BRG1)	19p13.2
	SMARCB1	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily b, member 1 (BAF47)	22q11.23
	SMARCC1	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily c, member 1 (BAF155)	3p21.31
	SMARCC2	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily c, member 2 (BAF170)	12q13.2
	SMARCD1	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily d, member 1 (BAF60A)	12q13.12
	SMARCD2	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily d, member 2 (BAF60B)	17q23.3
	SMARCD3	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily d, member 3 (BAF60C)	7q36.1

Table I. *Continued*

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Group	Gene symbol	Gene name	Ensembl genomic location
	SMARCE1	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily e, member 1 (BAF57)	17q21.2
Histone-modifying complexes	*ASH2L*	Ash2 (absent, small, or homeotic)-like (Drosophila)	8p11.23
	*DPY30**	Dpy-30 homolog (C. elegans)	2p22.3
	MEN1	Multiple endocrine neoplasias I	11q13.1
	MLL	Myeloid/lymphoid or mixed-lineage leukemia (trithorax homolog, Drosophila	11q23.3
	MLL2	Myeloid/lymphoid or mixed-lineage leukemia 2	12q13.12
	*MLL3**	Myeloid/lymphoid or mixed-lineage leukemia 3	7q36.1
	MLL4	Myeloid/lymphoid or mixed-lineage leukemia 4	19q13.12
	*MLL5**	Myeloid/lymphoid or mixed-lineage leukemia 5 (trithorax homolog, Drosophila	7q22.3
	NCOA6	Nuclear receptor coactivator 6 (ASC2)	20q11.22
	RBBP5	Retinoblastoma binding protein 5	1q32.1
	*WDR5**	WD repeat domain 5	9q34.2
Demethylases	*KDM5A*	Lysine (K)-specific demethylase 5A (RBP2)	12p13.33
	KDM5B	Lysine (K)-specific demethylase 5B (PLU1)	1q32.1
	KDM5C	Lysine (K)-specific demethylase 5C (SMCX)	Xp11.22
Polycomb proteins			
Polycomb repressive complex 1	*BMI1*	BMI1 polycomb ring finger oncogene	10p12.2
	CBX2	Chromobox homolog 2	17q25.3
	CBX4	Chromobox homolog 4	17q25.3
	CBX6	Chromobox homolog 6	22q13.1
	CBX8	Chromobox homolog 8	17q25.3
	PCGF2	Polycomb group ring finger 2 (MEL-18)	17q12
	PHC1	Polyhomeotic homolog 1 (Drosophila)	12p13.31
	PHC2	Polyhomeotic homolog 2 (Drosophila)	1p35.1
	PHC3	Polyhomeotic homolog 3 (Drosophila)	3q26.2
	RING1	Ring finger protein 1	6p21.32
	RNF2	Ring finger protein 2 (RING1B)	1q25.3
Polycomb repressive complex 2	*EED*	Embryonic ectoderm development	11q14.2
	EZH1	Enhancer of zeste homolog 1 (Drosophila)	17q21.2
	EZH2	Enhancer of zeste homolog 2 (Drosophila)	7q36.1
	SUZ12	Suppressor of zeste 12 homolog (Drosophila)	17q11.2
Cell-fate & development	*ALDH1A1*	Aldehyde dehydrogenase 1 family, member A1	9q21.13
	ALDH1A3	Aldehyde dehydrogenase 1 family, member A3	15q26.3
	CD44	CD44 molecule (Indian blood group)	11p13
	HOXA1	Homeobox A1	7p15.2
	HOXA2	Homeobox A2	7p15.2
	HOXA3	Homeobox A3	7p15.2
	HOXA4	Homeobox A4	7p15.2
	HOXA5	Homeobox A5	7p15.2
	HOXA6	Homeobox A6	7p15.2
	HOXA7	Homeobox A7	7p15.2
	HOXA9	Homeobox A9	7p15.2
	HOXA10	Homeobox A10	7p15.2
	HOXA11	Homeobox A11	7p15.2
	*HOXA13**	Homeobox A13	7p15.2
	HOXB1	Homeobox B1	17q21.32
	HOXB2	Homeobox B2	17q21.32
	HOXB3	Homeobox B3	17q21.32
	*HOXB4**	Homeobox B4	17q21.32
	HOXB5	Homeobox B5	17q21.32
	HOXB6	Homeobox B6	17q21.32
	HOXB7	Homeobox B7	17q21.32
	HOXB8	Homeobox B8	17q21.32
	HOXB9	Homeobox B9	17q21.32

Table I. *Continued*

Table I. *Continued*

Group	Gene symbol	Gene name	Ensembl genomic location
	HOXB13	Homeobox B13	17q21.32
	HOXC4	Homeobox C4	12q13.13
	HOXC5	Homeobox C5	12q13.13
	HOXC6	Homeobox C6	12q13.13
	HOXC8	Homeobox C8	12q13.13
	*HOXC9**	Homeobox C9	12q13.13
	HOXC10	Homeobox C10	12q13.13
	HOXC11	Homeobox C11	12q13.13
	*HOXC12**	Homeobox C12	12q13.13
	HOXC13	Homeobox C13	12q13.13
	HOXD1	Homeobox D1	2q31.1
	HOXD3	Homeobox D3	2q31.1
	*HOXD4**	Homeobox D4	2q31.1
	*HOXD8**	Homeobox D8	2q31.1
	HOXD9	Homeobox D9	2q31.1
	HOXD10	Homeobox D10	2q31.1
	HOXD11	Homeobox D11	2q31.1
	HOXD12	Homeobox D12	2q31.1
	HOXD13	Homeobox D13	2q31.1
	KLF4	Kruppel-like factor 4 (gut)	9q31.2
	MYC	v-myc myelocytomatosis viral oncogene homolog (avian)	8q24.21
	POU5F1	POU class 5 homeobox 1 (OCT4)	6p21.33
	SOX2	SRY (sex determining region Y)-box 2	3q26.33
	SOX4	SRY (sex determining region Y)-box 4	6p22.6
	SOX9	SRY (sex determining region Y)-box 9	17q24.3
Cell-cycle control	*BRCA1*	Breast cancer 1, early onset	17q21.31
	CDKN2A	Cyclin-dependent kinase inhibitor 2A (melanoma, p16, inhibits CDK4)	9p21.3
	CDKN2B	Cyclin-dependent kinase inhibitor 2B (p15, inhibits CDK4)	9p21.3
	CDKN2C	Cyclin-dependent kinase inhibitor 2C (p18, inhibits CDK4	1p32.3
	CDKN2D	Cyclin-dependent kinase inhibitor 2D (p19, inhibits CDK4)	19p13.2
	TP53	Tumor protein p53	17p13.1

phenotype. In particular, terminally-differentiated luminal phenotype is associated with a dramatic decrease (by 80% in ADH and 90% in DCIS) of *MME*, the gene coding for common acute lymphoblastic leukemia antigen (CALLA, also called CD10), a membrane metallopeptidase prevalently expressed in myoepithelium (22) (Figure 3). This finding is of relevance considering that myoepithelial cells control mammary gland homeostasis by forming a physical barrier between epithelial cells and the surrounding stroma, and by secreting paracrine mediators that inhibit tumor growth, invasion and angiogenesis (23). Furthermore, clinical evidence has indicated that the transition from *in situ* to invasive carcinoma is associated with the loss of myoepithelial layer and CD10 expression decreases in DCIS and is completely lost in invasive breast cancer (24). The down-regulation we observed already in ADH lesions (by 80% with respect to HN tissue) suggests that alteration of myoepithelium is a very early event in the pathological transformation of the mammary gland. Such alteration should be associated with the switch from the normal paracrine mechanism of action of ER-positive cells to an abnormal autocrine one as indicated by the observation that in both precursors, *ESR1* overexpression was paralleled by underexpression of *EGFR*, the gene coding for the growth factor receptor able to bind amphiregulin (the only EGFR ligand induced by estrogens). The constitutive expression of ER should make ADH- and DCIS-forming cells able to autocrinally-exploit the proliferative stimulus induced by estrogens and to by-pass the constraint of the dissociation between ER expression and cell proliferation. However, since none of the genes involved in cell-cycle control was differentially expressed in ADH or DCIS compared to HN tissue, it is conceivable that the shift from a non-dividing ER-positive luminal phenotype to a dividing ER-dependent phenotype was triggered but not yet completed.

Establishing the ER-dependent phenotype is associated with the overexpression of *FOXA1* and *GATA3* pioneer factors (Figure 3). Already overexpressed in ADH (by 32% and 54%

Table II. *Genes differentially expressed between atypical ductal hyperplasia (ADH) or ductal carcinoma in situ (DCIS) and histologically-normal (HN) tissue with an estimated false discovery rate (FDR) <0.1 (global model).*

Gene name	Gene symbol	ADH *versus* HN		DCIS *versus* HN	
		p-Value	% Variation*	*p*-Value	% Variation
Actin, alpha 2, smooth muscle	*ACTA2*	0.000037	–62	0.000005	–72
Actin-like 6A	*ACTL6A*			0.002330	+59
Chromobox homolog 8	*CBX8*	0.002807	–40	0.002612	–40
CD24 molecule	*CD24*	0.000912	+32	0.000703	+33
Epidermal growth factor receptor	*EGFR*	0.000089	–47	0.000014	–55
Epithelial cell adhesion molecule	*EPCAM*	0.009415	+57	0.003975	+64
Estrogen receptor 1	*ESR1*	0.006203	+56	0.001160	+69
Enhancer of zeste homolog 2	*EZH2*	0.013528	+127	0.000224	+207
Forkhead box A1	*FOXA1*			0.001693	+93
GATA binding protein 3	*GATA3*	0.002335	+54	0.000180	+70
Homeobox A1	*HOXA1*	0.005013	–57	0.002426	–62
Homeobox A2	*HOXA2*	0.002912	–65	0.000572	–78
Homeobox A4	*HOXA4*	0.002221	–40	0.001919	–41
Homeobox A5	*HOXA5*	0.000196	–59	0.000013	–73
Homeobox A7	*HOXA7*	0.001813	–30	0.000661	–34
Homeobox A9	*HOXA9*	0.014944	–68	0.002681	–87
Keratin 18	*KRT18*			0.004565	+65
Keratin 19	*KRT19*			0.002157	+93
Membrane metallo-endopeptidase	*MME*	0.000006	–80	0.000001	–90
mucin 1, cell surface associated	*MUC1*	0.048278	+88	0.001816	+148
SRY (sex determining region Y)-box 4	*SOX4*	0.021910	+40	0.000275	+70
SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily e, member 1	*SMARCE1*			0.005343	+42

*Relative variation was calculated as (ADH-HN)/HN or (DCIS-HN)/HN.

with respect to HN tissue), *FOXA1* and *GATA3* expression greatly increased in DCIS (by 93% and 70%, respectively). In agreement with the notion that GATA3 and ER enhance each other's transcription (8), *ESR1* and *GATA3* expression increased in a parallel manner both in ADH (by 56% and 54%, respectively) and in DCIS (by 69% and 70%, respectively).

The concomitant overexpression of *FOXA1*, *GATA3* and *ESR1* is in accordance with the mechanism of action proposed for pioneer factors (6). Due to their ability to associate directly with condensed chromatin independently of and prior to any other transcription factor, pioneer factors allow the binding of other transcription factors (in this case ER) that cannot bind on their own. Experimental studies have demonstrated that FOXA1 moves slowly along chromatin structure scanning chromatin for enhancers with forkhead motifs (25). Thereafter, it triggers transcriptional competency of enhancers through cooperation with additional pioneer factors including GATA family members (26), thus explaining the co-recruitment of GATA3 and FOXA1 to ER *cis*-regulatory elements.

The binding of FOXA to chromatin sites, however, depends on DNA and histone modifications (especially, DNA de-methylation, and mono- and di-methylation of histone H3 at lysine 4), required to stabilize pioneer factor binding and allow the subsequent recruitment of other transcription factors. Chromatin remodeling and histone modifications are under the control of Trithorax and Polycomb maintenance proteins organized as multimeric complexes. Two distinct classes of chromatin-modifying enzymes have been identified: one disrupts chromatin structure in an ATP-dependent manner; the other acts through covalent post-translational modification of histone proteins. Several reports suggest that ATP-dependent chromatin remodeling complexes influence ligand-activated nuclear receptor activity (27). In particular, the switching-defective (SWI)/sucrose non-fermnting (SNF) chromatin remodeling complex, a large multiprotein complex, formed by Brahma-related gene 1 (BRG1) as the central catalytic ATPase and several BRG1-associated factors (BAFs), interacts with ER through the direct or indirect interaction of one or more BAF subunits (28). Among the genes coding for SWI/SNF complex components only two, *ACTL6A* and *SMARCE1*, were differentially expressed (overexpressed) in our study. Remarkably, the genes, coding for BAF53A and BAF57 subunit respectively, were differentially expressed only in DCIS (Figure 4), suggesting that in the establishment of ER-dependent autocrine phenotype, overexpression of these subunits is a second step after constitutive overexpression of ER. Studies

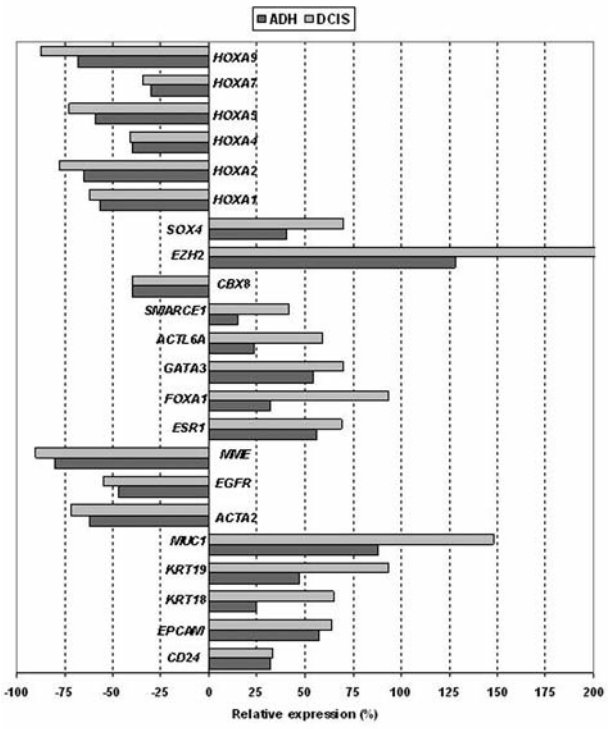

Figure 1. *The 22 genes differentially expressed between histologically-normal (HN) tissue and precursor lesions [atypical ductal hyperplasia (ADH) or ductal carcinoma in situ (DCIS)] with an estimated false discovery rate (FDR) <0.1 (global model). Differential gene expression is represented as relative to NH expression value.*

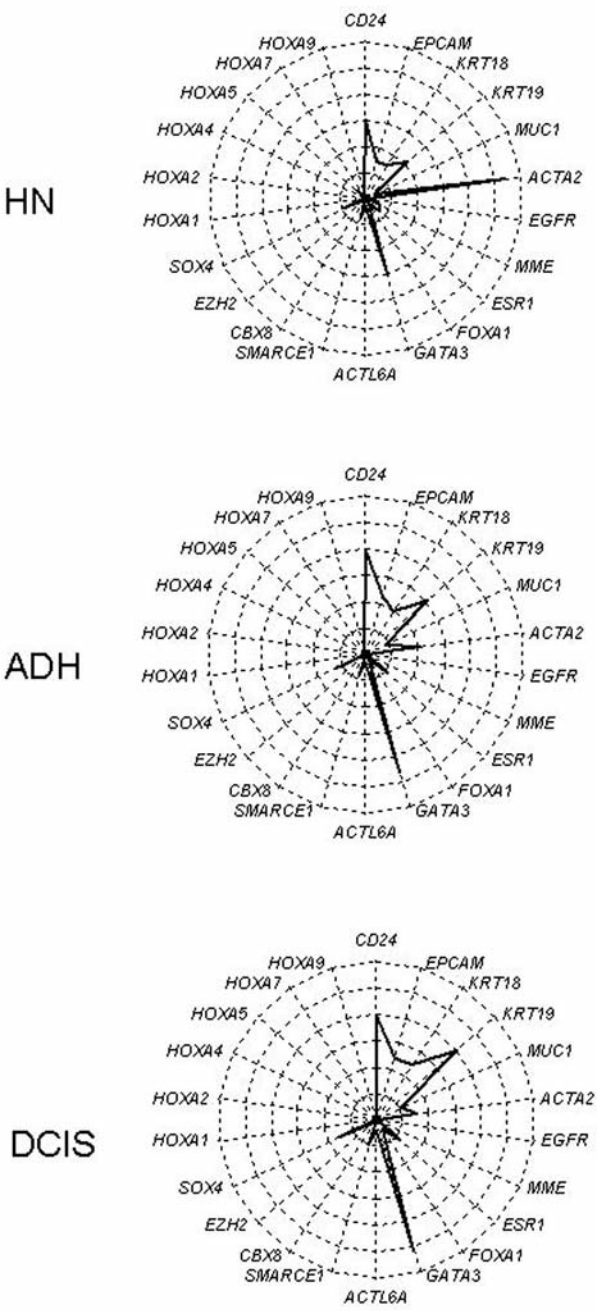

Figure 2. *Radar charts of the 22 genes differentially expressed between histologically-normal (HN) tissue and precursor lesions [atypical ductal hyperplasia (ADH) or ductal carcinoma in situ (DCIS)]. The center of the chart corresponds to no gene expressions while the edges correspond to a gene expression value of 3000.*

aimed at investigating the functional role of SWI/SNF chromatin remodeling complex in ER-mediated transcriptional activity have demonstrated that the complex is recruited to estrogen-responsive promoters by the interaction of its BAF53 subunit (29) and that BAF57 levels are critical for the correct transmission of estrogen-dependent signals: the reduction of BAF57 expression specifically inhibited the transcription of ER target genes and blocked estrogen-dependent cell proliferation (30). In this scenario, the constitutive synthesis of BAF57 may contribute to the establishment and maintenance of autocrine ER-mediated signaling.

With regard to the genes coding for proteins belonging to the Polycomb group, only *CBX8* and EZH2 proved to be differentially expressed (Figure 4). Coding for an essential component of polycomb repressive complex 1 (PRC1), *CBX8* was underexpressed (by 40% in both types of lesion), while *EZH2*, which codes for the catalytic subunit of PRC2, was progressively overexpressed. With respect to HN tissue, *EZH2* expression increased of by 127% in ADH and by 207% in DCIS. Originally discovered in Drosophila, where they proved to silence the expression of key developmental regulators such as homeobox genes by epigenetic modification, PRC1 and PRC2 complexes work cooperatively. Through EZH2, PRC2 achieves covalent di- and tri-methylation of histone H3 at lysine 27, providing a specific docking site for proteins harboring a chromobox domain and helping the recruitment of PRC1 complex to chromatin (31). After binding to chromatin, PRC1 catalyzes the mono-ubiquitylation of lysine

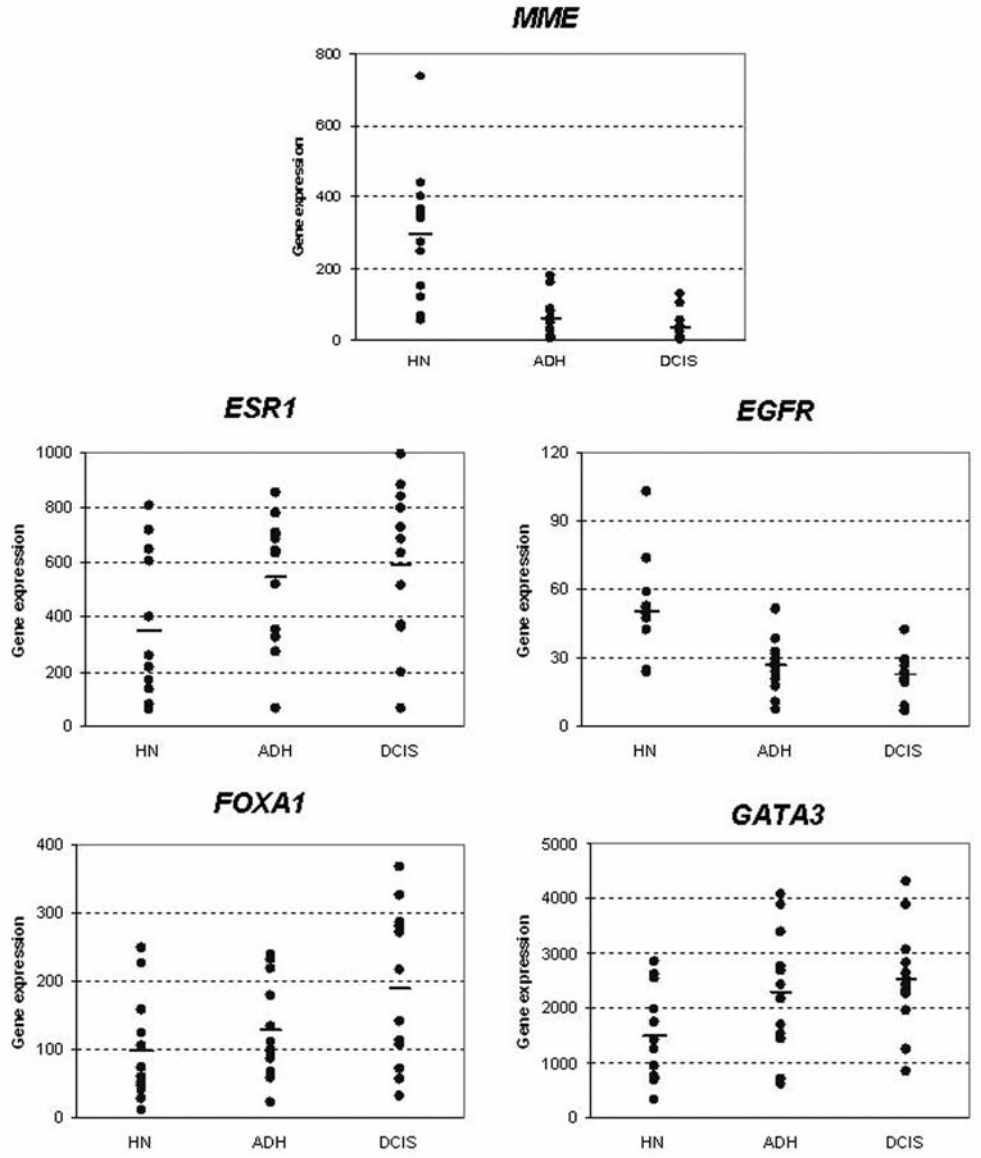

Figure 3. *Scatter plots of the genes coding for CD10 (MME), estrogen receptor (ESR1), epidermal growth factor receptor (EGFR) and pioneer factors [forkhead box A1 (FOXA1) and GATA binding protein 3 (GATA3)] in atypical ductal hyperplasia (ADH), ductal carcinoma in situ (DCIS) and histologically-normal (HN) tissue.*

119 at histone H2A and blocks the elongation of RNA polymerase II, leading to repression of gene transcription. The finding that, ever in ADH lesions, *CBX8* expression was significantly reduced suggests the disruption of gene silencing an early and pivotal step in the pathological transformation. In our case series, *CBX8* underexpression was associated with *EZH2* overexpression, in agreement with recent studies showing a low expression level of EZH2 in morphologically-normal lobules and a progressive increase in protein expression with increasing severity of epithelial atypia (32). In addition, in patients with invasive breast carcinoma, high concentrations of *EZH2* transcript and protein have been reported to be associated with tumor cell proliferation and metastatic potential (33). Giving the general belief according to which EZH2 expression leads to chromatin hypermethylation and consequently inhibition of the target gene inhibition, the observation that EZH2 functions as a gene transcriptional activator should appear surprising. However, experimental evidence indicated that EZH2 methyltransferase activity is required for the activation and maintenance of the activated state of some genes specifically involved in cell proliferation (34). In particular, EZH2 was proven to interact

Table III. *F-Test on the coefficients obtained from a model for repeated measures with random effect*.

	ADH *versus* HN		DCIS *versus* HN	
Gene symbol	*p*-Value	FDR	*p*-Value	FDR
ACTA2	0.000037	**0.002192**	0.000005	**0.000311**
ACTL6A	0.167542	0.478108	0.002330	**0.016507**
ACTL6B	0.735137	0.870333	0.989605	0.989605
ALDH1A1	0.980871	0.985655	0.755040	0.833393
ALDH1A3	0.180875	0.485990	0.111208	0.236570
ARID1A	0.158738	0.476762	0.119606	0.237185
ASH2L	0.186158	0.485990	0.020677	**0.078039**
BMI1	0.143748	0.468645	0.019633	**0.078039**
BPTF	0.831872	0.939274	0.186408	0.326034
BRCA1	0.495855	0.763356	0.041724	0.135602
BRD7	0.694858	0.870333	0.714550	0.818577
CARM1	0.906050	0.981554	0.083669	0.208283
CBX2	0.377238	0.731390	0.914240	0.930140
CBX4	0.022997	0.149482	0.908661	0.930140
CBX6	0.009231	**0.078685**	0.096117	0.224914
CBX8	0.002807	**0.034069**	0.002612	**0.016507**
CD24	0.000912	**0.021337**	0.000703	**0.008224**
CD44	0.113842	0.416236	0.186703	0.326034
CDH1	0.350744	0.707535	0.390979	0.551139
CDKN2A	0.065775	0.295988	0.018479	**0.078039**
CDKN2B	0.834201	0.939274	0.066617	0.189425
CDKN2C	0.063512	0.295988	0.425558	0.572303
CDKN2D	0.970146	0.985655	0.675109	0.816083
EED	0.842939	0.939274	0.766411	0.838038
EGFR	0.000089	**0.003481**	0.000014	**0.000397**
EPCAM	0.009415	**0.078685**	0.003975	**0.023253**
ESR1	0.006203	**0.060476**	0.001160	**0.012343**
ESR2	0.702049	0.870333	0.608215	0.799222
EZH1	0.050376	0.256259	0.059663	0.174514
EZH2	0.013528	0.105522	0.000224	**0.004366**
FOXA1	0.243639	0.537844	0.001693	**0.016039**
GATA3	0.002335	**0.034069**	0.000180	**0.004207**
HOXA1	0.005013	**0.053320**	0.002426	**0.016507**
HOXA2	0.002912	**0.034069**	0.000572	**0.008224**
HOXA3	0.334198	0.685986	0.904636	0.930140
HOXA4	0.002221	**0.034069**	0.001919	**0.016039**
HOXA5	0.000196	**0.005722**	0.000013	**0.000397**
HOXA6	0.642423	0.854131	0.191550	0.329578
HOXA7	0.001813	**0.034069**	0.000661	**0.008224**
HOXA9	0.014944	0.109279	0.002681	**0.016507**
HOXA10	0.218162	0.517805	0.156274	0.290223
HOXA11	0.482436	0.763333	0.218412	0.370351
HOXB1	0.464893	0.763333	0.853370	0.899498
HOXB2	0.217549	0.517805	0.311848	0.463339
HOXB3	0.234710	0.528098	0.422679	0.572303
HOXB5	0.979372	0.985655	0.302849	0.463339
HOXB6	0.033189	0.204376	0.099977	0.229123
HOXB7	0.406328	0.731390	0.118651	0.237185
HOXB8	0.722478	0.870333	0.732551	0.820323
HOXB9	0.394783	0.731390	0.614832	0.799222
HOXB13	0.331211	0.685986	0.232300	0.388274
HOXC10	0.714194	0.870333	0.665868	0.816083
HOXC11	0.144433	0.468645	0.027955	**0.096199**
HOXC13	0.981798	0.985655	0.655357	0.816083
HOXC4	0.890448	0.973668	0.116397	0.237185
HOXC5	0.515695	0.783588	0.367217	0.530424
HOXC6	0.631415	0.852577	0.833812	0.886873
HOXC8	0.734120	0.870333	0.355871	0.520461
HOXD1	0.598306	0.841389	0.070236	0.191108
HOXD3	0.401018	0.731390	0.103791	0.229123
HOXD9	0.469476	0.763333	0.088071	0.211247
HOXD10	0.110113	0.415588	0.288929	0.456821
HOXD11	0.599316	0.841389	0.459491	0.610914
HOXD12	0.965336	0.985655	0.077708	0.202041
HOXD13	0.982543	0.985655	0.685433	0.816083
KDM5A	0.841434	0.939274	0.708293	0.818577
KDM5B	0.158921	0.476762	0.006725	**0.034211**
KDM5C	0.736436	0.870333	0.671381	0.816083
KLF4	0.203140	0.516683	0.736187	0.820323
KRT14	0.043327	0.241393	0.018756	**0.078039**
KRT17	0.108228	0.415588	0.048027	0.147872
KRT18	0.233249	0.528098	0.004565	**0.025434**
KRT19	0.092143	0.385028	0.002157	**0.016507**
KRT5	0.058066	0.283071	0.008226	**0.040103**
KRT6A	0.383854	0.731390	0.236647	0.389968
KRT6B	0.446276	0.763333	0.686448	0.816083
KRT7	0.599435	0.841389	0.422024	0.572303
KRT8	0.213608	0.517805	0.020441	**0.078039**
MEN1	0.709029	0.870333	0.088471	0.211247
MLL	0.072341	0.313478	0.133003	0.255104
MLL2	0.475838	0.763333	0.308339	0.463339
MLL4	0.185783	0.485990	0.108404	0.234875
MME	0.000006	**0.000693**	0.000001	**0.000146**
MUC1	0.048278	0.256259	0.001816	**0.016039**
MYC	0.546754	0.809749	0.636230	0.809118
NCOA3	0.611265	0.841389	0.046732	0.147776
NCOA6	0.252257	0.546556	0.054358	0.163075
NCOR1	0.609651	0.841389	0.876938	0.916087
NCOR2	0.582119	0.841389	0.264426	0.429692
PBRM1	0.699339	0.870333	0.032538	0.108771
PCGF2	0.389410	0.731390	0.103336	0.229123
PGR	0.423234	0.750278	0.180500	0.324901
PHC1	0.633967	0.852577	0.312853	0.463339
PHC2	0.881520	0.972999	0.703731	0.818577
PHC3	0.950293	0.985655	0.146012	0.275538
POU5F1	0.483362	0.763333	0.981441	0.989605
RBBP4	0.035685	0.208760	0.009083	**0.042506**
RBBP5	0.221284	0.517805	0.114781	0.237185
RBBP7	0.985655	0.985655	0.019235	**0.078039**
RING1	0.526078	0.789116	0.823106	0.883518
RNF2	0.148204	0.468645	0.083660	0.208283
SMARCA1	0.144555	0.468645	0.621617	0.799222
SMARCA2	0.694337	0.870333	0.690532	0.816083
SMARCA4	0.104189	0.415588	0.022014	**0.080487**
SMARCB1	0.164610	0.478108	0.382857	0.546272
SMARCC1	0.489316	0.763333	0.067999	0.189425
SMARCC2	0.766582	0.888021	0.412621	0.572303
SMARCD1	0.469393	0.763333	0.275145	0.440986
SMARCD2	0.930510	0.985655	0.177032	0.323637
SMARCD3	0.733672	0.870333	0.806461	0.873666
SMARCE1	0.289397	0.615626	0.005343	**0.028415**
SOX2	0.763399	0.888021	0.720628	0.818577
SOX4	0.021910	0.149482	0.000275	**0.004590**
SOX9	0.374834	0.731390	0.130475	0.254427
SUZ12	0.186919	0.485990	0.023738	**0.084162**
TP53	0.141651	0.468645	0.076899	0.202041
VIM	0.477471	0.763333	0.308965	0.463339

FDR, False discovery rate, estimated FDR <0.10 are shown in bold.

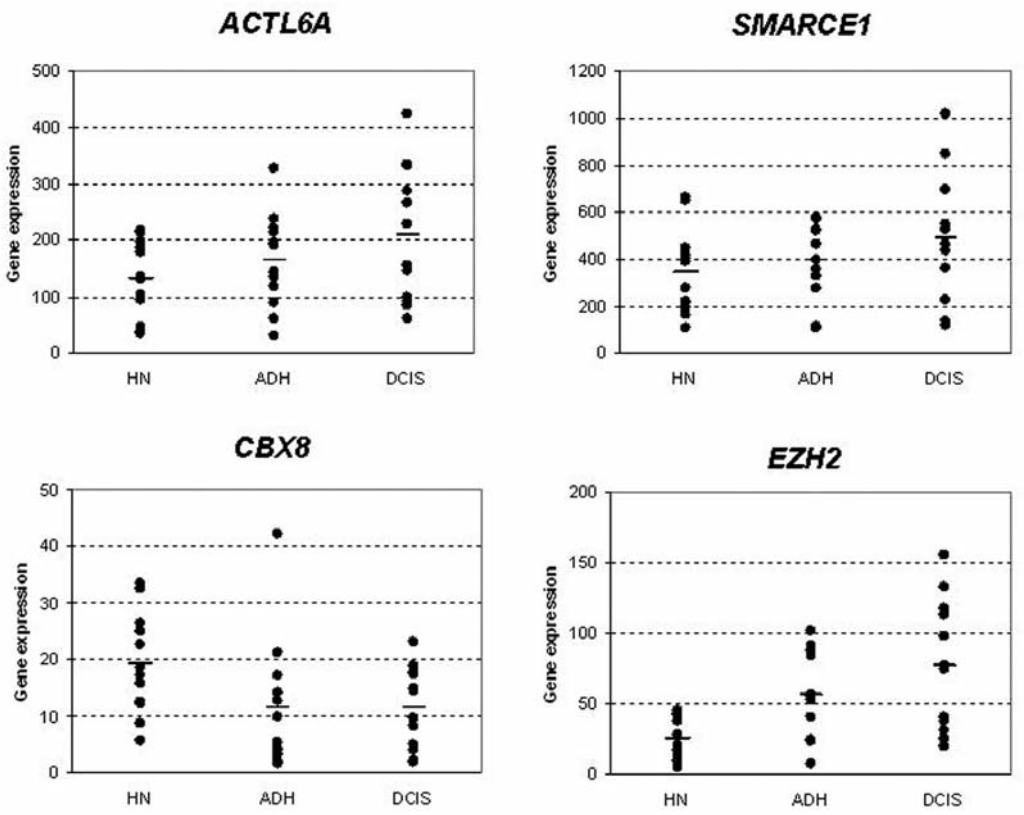

Figure 4. *Scatter plots of the genes coding for Trithorax [Actin-like 6A (ACTL6A) and SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily e, member 1 (SMARCE1)], and Polycomb complex components [chromobox homolog 8 (CBX8) and enhancer of zeste homolog 2 (EZH2)] in atypical ductal hyperplasia (ADH), ductal carcinoma in situ (DCIS) and histologically-normal (HN) tissue.*

directly with ER and β-catenin, thus connecting estrogen and Wnt signaling pathways in promoting cell-cycle progression (35). Involvement of EZH2 in the promotion of neoplastic transformation of mammary epithelial cells is further supported by experimental evidence in transgenic mice, demonstrating that *Ezh2* overexpression dysregulates mammary epithelial cell memory, reduces cell plasticity and causes intra-ductal epithelial hyperplasia (36). Notably, EZH2-induced hyperplasia was predominantly composed of differentiated luminal cells which express ER, cytokeratin-18, E cadherin and high levels of GATA3, the latter known to be essential for specification and maintenance of the luminal cell fate in the mammary gland (37). In further agreement with these findings, we observed (Figure 5) progressive overexpression (by 40% in ADH and 70% in DCIS) of *SOX4*, the gene coding for a commitment factor that induces the luminal differentiation of epithelial cells during postnatal mammary gland development (38).

In our study, the differentiated luminal ER-dependent phenotype was associated with the selective underexpression of the entire *HOXA* cluster gene. In fact, of the 39 human *HOX* genes, only *HOXA* cluster components were statistically significantly down-regulated in both precursors (Figure 5). In particular, *HOXA2*, *HOXA5* and *HOXA9* showed the most pronounced decrease with a reduction in DCIS of 78%, 73% and 87%, respectively. The finding that only HOXA cluster expression was dysregulated in pathological tissues was not surprising, since genetic studies have demonstrated that in human breast cancer, the *HOXA* gene cluster undergoes a selective epigenetic inactivation because of DNA hypermethylation (39).

Some recent studies in non-cancerous cells have provided evidence of a mechanistic link between Polycomb activity and tumor-associated hypermethylation events (40, 41), and indicated that at least in undifferentiated cells, Polycomb complexes have the potential to drive target genes towards silencing or activation (42). The transition between alternative modes of Polycomb regulation would require for specific signaling as, for example, ovarian steroid hormones, and recruitment of additional transcriptional activators such as ligand-activated nuclear receptors. According to a similar scenario, it is conceivable that in ADH- and DCIS-forming cells, *HOXA* genes are aberrantly hypermethylated by EZH2 overexpression and switched off.

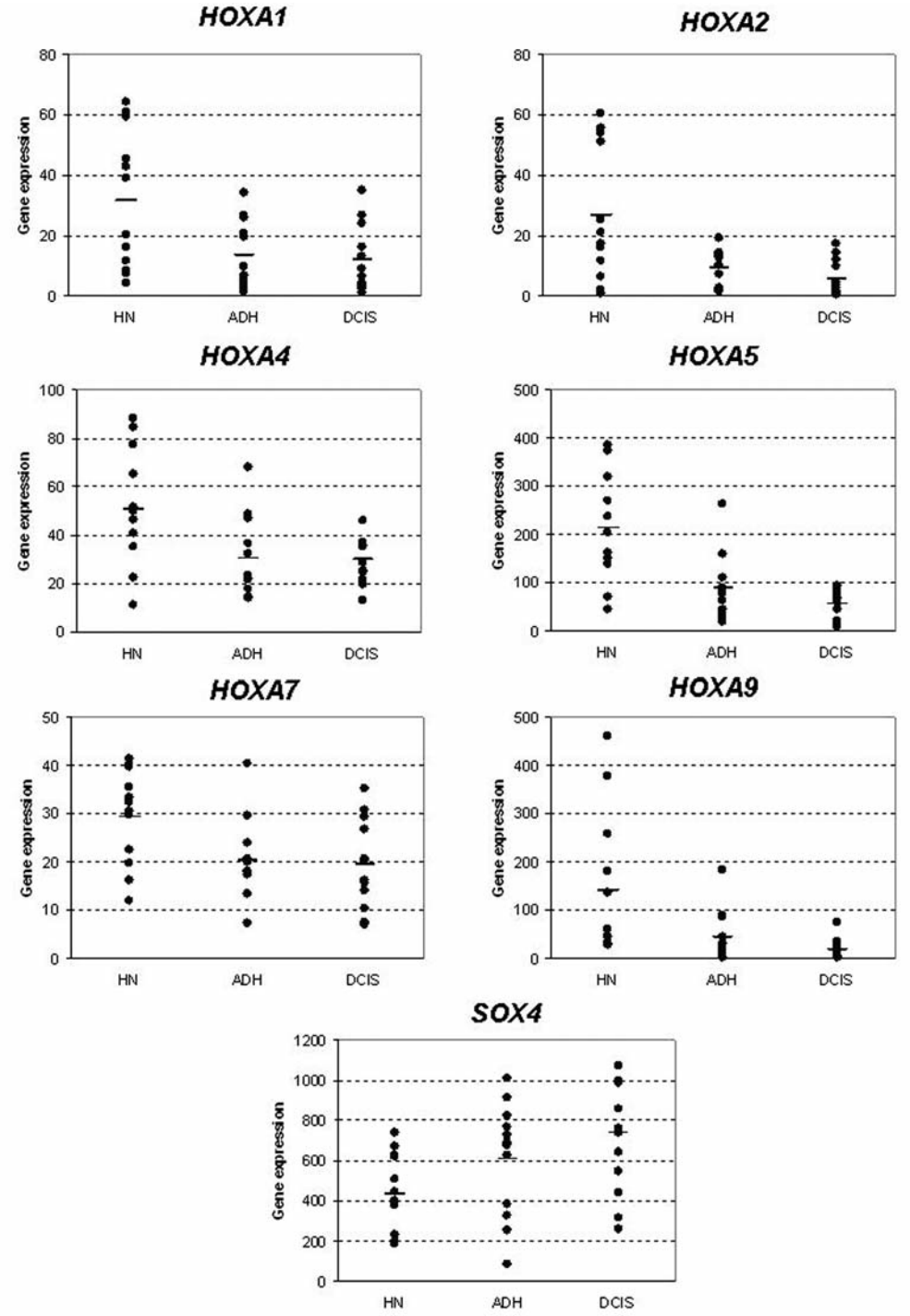

Figure 5. *Scatter plots of the genes coding for Homeobox A (HOXA) cluster genes and SRY (sex determining region Y)-box 4 (SOX4) in atypical ductal hyperplasia (ADH), ductal carcinoma in situ (DCIS) and histologically-normal (HN) tissue.*

Although the mechanisms triggering initiation of breast carcinogenesis have not been completely elucidated, recent studies have indicated dysregulation of microRNAs as one of the possible causes for pathological transformation. Indeed, accumulating evidence has indicated microRNAs, the small non-coding RNAs that regulate gene expression by inhibiting translation or promoting degradation of specific target mRNA, as being involved in DNA methylation and histone

modifications. Several microRNAs have been proven to regulate EZH2 expression (43, 44) and one of them (miR-26a) also targeted the *ESR1* gene (45), thus providing a possible explanation for the co-overexpression of EZH2 and ER protein observed in ER-positive breast cancer cells. In addition, *SOX4* overexpression was recently associated with the epigenetic silencing of the gene coding for miR-129-2 (46). Notably, among the experimentally-verified targets of miR-129-2 (47), there is also *ESR1*, thus providing an additional explanation for the concomitant overexpression of *SOX4* and *ESR1*.

According to the role of DNA methylation and histone modifications in the control of mammary gland development and differentiation, an increasing body of evidence indicates disruption in the epigenetic control of gene transcription as a possible cause for pathological transformation and tumor initiation. Collectively, our findings seem to support this hypothesis and indicate that in TDLU-forming cells, disruption of epigenetic control is associated with loss of cell identity and acquisition of a constitutive estrogen-dependent, terminally-differentiated luminal phenotype, aimed to exploit, in an autocrine manner, the proliferative stimulus induced by estrogens present in the microenvironment. Indeed, we found a close association among Polycomb complex components overexpression, silencing of *HOXA* cluster gene, and overexpression of the genes involved in estrogen signaling (specifically, *FOXA1* and *GATA3* pioneer factors, and *ESR1*).

There is no doubt that the present *in silico* study suffers from the limitation common to the majority of studies involving gene expression profiling, that is, the lack of validation of the observed mRNA modulations at the protein level. However, our results raise interesting suggestions regarding the initial steps of breast tumorigenesis and are valuable for further investigation principally aimed to elucidate the tumor-promoting function of intralobular stroma on TDLU epithelial components, and in particular, how estrogens produced by the putatively normal microenvironment affect epithelial cell identity.

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