Despite increasing progress, breast cancer remains the most common oncologic disease among women. This fact emphasizes the need for effective preventive intervention. Accumulating evidence indicates that epigenetic control of gene expression plays a fundamental role in mammary tissue homeostasis, and suggests that alterations in such a fine control may affect epithelial cell identity and trigger neoplastic transformation. Therefore, understanding the biological basis of breast cancer initiation is of paramount importance to prevent pathologic alterations leading to the emergence of premalignant lesions that potentially could evolve into invasive cancer.

In this overview, we described and discussed some recent findings from two early breast cancer precursors, hyperplastic enlarged lobular unit (HELU) and atypical ductal hyperplasia (ADH), in which we investigated the pattern of expression of a panel of genes involved in the control of cell identity and mammary gland remodeling, compared to the corresponding histologically normal tissue. Collectively, findings suggest that the changes in cell identity associated with HELU and ADH development are likely due to alterations in the epigenetic control of gene transcription and that a possible cause for the occurrence of these alterations could be an abnormal and/or persistent ovarian hormone signaling from surrounding microenvironment. Since, unlike genetic alterations, epigenetic changes are potentially reversible, in the last part of the overview, some epigenome-targeting strategies, potentially useful for cancer prevention are proposed and discussed.

Keywords: breast cancer precursors; epithelial cell identity; stromal compartment

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An emerging area of interest in cancer research is the investigation of tumor microenvironment, its cellular and molecular components, and the reciprocal interactions between tumor cells and the supporting stromal cells.
remodeling through a continuous and dynamic cross talk with epithelial cells. In addition, a growing body of evidence has demonstrated that alterations occurring in the epithelium-stroma interplay may contribute to tumor initiation and promotion. A representative case of the detrimental effects caused by disruption of the structural and functional relationship between epithelial tissue and supporting stromal cells is breast cancer, where the significant role of stromal compartment on tumor initiation and progression has increasingly been recognized [1-4]. Likewise, since the amount of stroma within the primary tumor has proved to have prognostic value, the tumor-stroma ratio has recently been proposed as prognostic factor in patients with early breast cancer and, in particular, in those with a triple-negative cancer [5, 6]. However, despite the increasing awareness that stroma contributes to tumor development and progression, scanty attention has been devoted to elucidate the interplay between stroma and premalignant lesions or to understand how early stroma modifications may affect normal epithelium, disrupt its architecture and trigger neoplastic transformation. Indeed, only few papers are present in literature and mainly focus on the mechanisms underlying the progression from carcinoma in situ to invasive cancer [7, 8].

According to the model proposed for mammary carcinogenesis, normal epithelial cells progressively accumulate (epi) genetic alterations that result in a series of histologically identifiable, even if non-obligate, cancer precursors [9]. In relation to the glandular structure (duct or lobule) involved in the tumorigenic process, pathological stages roughly include flat epithelial atypia (FEA) or hyperplastic enlarged lobular unit (HELU), atypical ductal or lobular hyperplasia (ADH, ALH), preinvasive ductal or lobular carcinoma in situ (DCIS, LCIS), and invasive ductal or lobular carcinoma (IDC, ILC). IDCs account for 80-85% of all diagnosed breast cancers and about two-thirds of them are characterized by an estrogen receptor(ER)-positive status. ILCs account for about 10-15% of total cases and are mostly ER-positive [10, 11].

Although benign breast diseases are non-obligate cancer precursors and only a small proportion of them actually progress to invasive cancer, they have been proposed as a potential valuable risk factor to be thoroughly investigated and monitored. Observational studies have indicated that FEA and HELU frequently coexist with other proliferative lesions including ADH and low-grade DCIS, suggesting their occurring as the first step in a biological continuum toward breast cancer [12]. In fact, clinical evidence indicated that, compared to the general population, women harboring ADH and ALH have a 3.5 to 5 fold increased risk of developing invasive cancer whereas the increased relative risk ups to 9 fold for LCIS and 12 fold for DCIS [13]. Because genetic and histopathological evidences support that most, if not all, invasive carcinomas derive from a previous preinvasive lesion [14], improving our knowledge about breast cancer initiation is of paramount importance to prevent pathologic alterations leading to the emergence of premalignant lesions that potentially could evolve into invasive cancer [15]. In particular, it is important the identification of the molecular events that mark the turning point toward neoplastic transformation.

Among the early events required for the neoplastic transformation of a mammary epithelial cell, there are perturbations in cell identity. This term refers to distinguishable and heritable features that characterize every cell within a multicellular organism. Indeed, the tissue-specific profile depends on a genetically encoded unidirectional program, according to which, stem cells lose their developmental potential and narrow down their plasticity into a particular differentiated cell type. To preserve tissue homeostasis it is essential to safeguard individual cell identity so that, at each DNA replication and mitosis, daughter cells could retain the phenotype of the parental cell (cell memory). Preservation of cell identity depends on a class of proteins that are collectively termed maintenance proteins [16] and act regulating gene expression in a somatically heritable but DNA-independent (i.e., epigenetically) manner. Maintenance proteins consist of heterogeneous proteins that primarily work on DNA methylation, histone modification and chromatin remodeling. The best-characterized maintenance proteins are those belonging to Trithorax (TrxG), and Polycomb (PcG) groups [17]. Originally discovered in Drosophila, where they exert an antagonist role on the transcription of homeobox genes during embryo development, TrxG and PcG proteins have proved to act as transcriptional activators or repressors depending on the multi-protein complex in which they are comprised in other organisms including human.

To elucidate the molecular events associated with breast cancer initiation, in a recent study [18] we interrogated some IDC precursors, namely simple hyperplasia (SH), ADH and DCIS, from a series of women with an ER-positive IDC. Specifically, we investigated the pattern of expression of a panel of 369 genes involved in the control of cell identity and mammary gland remodeling, and we compared it to the corresponding histologically normal tissue. Interestingly, we found that while no gene was differentially expressed between SH and normal tissue, ADH and DCIS were associated with an increasing panel of genes, which proved to be differentially expressed, in a statistically significant manner, compared to normal tissue. Noteworthy, the absence of differentially expressed genes between SH and normal tissue suggests the transition SH/ADH as the crucial turning point toward the pathological transformation whereas the observation that all genes found differentially expressed in ADH were differentially expressed also in
DCIS, corroborates the hypothesis that ADH is the biological precursor of DCIS.

Compared to normal tissue, ADH and DCIS are characterized by a terminally differentiated luminal phenotype. In fact, they overexpress genes associated with luminal differentiation (EPCAM, GATA3) and underexpress genes coding for markers associated with basal phenotype (ACTA2, EGFR, KRT17, and MME). Besides genes specifically associated with luminal differentiation, ADH and DCIS differentially express some genes encoding proteins involved in cell fate decision (JAG2, SOX10), embryonic development (HOXA4, HOXA5, and HOXA9), epigenetic control of gene transcription (CBX8, EZH2), and canonical and non-canonical TGF-β signaling pathways (FOXC1, ID2, SNAI2, TGFB3, and WNT5B). Among the genes that, respect to ADH, specifically portray DCIS there are CCNB1, CCNB2, MKI67 and ER1, which code respectively for cyclin B1, cyclin B2, the proliferation-associated antigen Ki67 and ERα, and proved to be overexpressed compared to normal tissue. The finding that, in DCIS, terminal luminal differentiation (indicated by the concomitant overexpression of ESR1 and underexpression of EGFR) is associated with cell proliferation (indicated by CCNB1, CCNB2 and MKI67 overexpression) is particularly interesting because in apparent contrast with the well-recognized principle that, in normal luminal cells, the expression of estrogen receptor and cell proliferation are antithetic processes [19]. In other words, normal luminal ER-positive/EGFR-negative cells do not proliferate but act as “sensor” and respond to estrogens from surrounding microenvironment by producing growth factors that paracrinally induce adjacent ER-negative/EGFR-positive cells to proliferate [20].

Our findings indicate that DCIS development is associated with disruption of the mechanism governing the dissociation between ER expression and cell proliferation, and acquisition of a constitutive estrogen-dependent phenotype [21] aimed to exploit autocrinally the proliferative stimulus induced by estrogens from surrounding microenvironment, providing a plausible explanation for the high risk of DCIS to progress toward invasive cancer. Notably, the acquisition of the constitutive estrogen-dependent phenotype is associated with the dramatic decrease (in our study, 90% in DCIS compared to normal tissue) of MME, the gene coding for CD10, a membrane metallopeptidase prevalently expressed in myoepithelium [22]. This finding has great biological relevance considering that myoepithelial cells control mammary gland homeostasis by forming a boundary, which physically separates epithelial cells from the surrounding stroma, and secreting paracrine mediators that inhibit tumor growth and angiogenesis, thus preventing tumor cell invasion [23]. Clinical evidence corroborates this notion and indicates structural alterations of the myoepithelial layer and a decreased expression of CD10 as associated with the in-situ-to-invasive transition [24]. Since the underexpression of MME was observed also in SH and ADH lesions, it is conceivable suppose the alteration of myoepithelium as a very early event in the pathological transformation of the mammary gland.

Our analysis also indicated that the acquisition of the constitutive estrogen-dependent phenotype is closely associated with overexpression of FOXA1 and GATA3, which encode respectively the pioneer factor forkhead box A1 and the transcription factor GATA binding protein 3, thus supporting the hypothesis that perturbations of cell identity are under epigenetic control. Already overexpressed in ADH, even not in a statistically significant manner, FOXA1 and GATA3 greatly increase their expression in DCIS. Furthermore, in agreement with the notion that Gata-3 and ER enhance each other’s transcription [25], we found that ESR1 and GATA3 expression increased in a parallel way. Notably, the concomitant overexpression of FOXA1, GATA3 and ESR1 is in accord with the model proposed to explain the mechanism of action of FoxA1 pioneer factor in mammary ductal morphogenesis [26]. Originally described as crucial transcriptional components in development and differentiation, pioneer factors have recently proved to act as mediators of nuclear receptor function under both normal and pathologic conditions [27]. In particular, FoxA1 has proved to be a master regulator of ER activity owing to its ability to bind simultaneously to DNA and core histones, rearrange chromatin structure and recruit ER to target gene promoters [28]. The pioneer capability of FoxA1 is due to the presence of a so-called forkhead box domain, a variant of the helix-turn-helix structure that has two large loops, giving it the appearance of a “winged helix”. Experimental studies have demonstrated that FoxA1 moves slowly along the chromatin scanning it for enhancers with forkhead motifs. Upon finding a forkhead motif, the central helix-turn-helix of FoxA1 directly interacts with the major groove of the DNA while each wing interacts with minor grooves adjacent to the target sequence. This particular conformation stabilizes the interaction with the DNA [29] and triggers the transcriptional competency of the enhancer, in cooperation with additional transcription factors such as GATA family members [30]. Among the members of GATA family, Gata-3 has proved to be essential for mammary gland development and maintenance of the terminal differentiation of the luminal lineage in adults [31]. In particular, Gata-3 has proved to be recruited with FoxA1 to ER cis-regulatory elements, where it enhances the transcription of target genes [25]. Notably, experimental studies have also revealed that Gata-3 is able to bind to FOXA1 promoter region and induce its expression [32].
Our analysis indicates that the overexpression of GATA3, FOXA1 and ESR1 is associated with that of ACTL6A and EZH2, which code respectively for a regulatory component of ATP-dependent chromatin-remodeling complex (BaF53A) and the inducible catalytic subunit of Polycomb repressive complex 2 (Ezh2) providing evidence for a relationship between cell identity perturbations and dysfunction in the epigenetic control of gene transcription. In particular, experimental studies have provided evidence that EZH2 overexpression dysregulated mammary epithelial cell memory, reduces cell plasticity, disrupts ductal morphogenesis and promotes epithelial hyperplasia in transgenic mice [33]. Remarkably, studies indicated that EZH2-induced hyperplasia was predominantly composed of differentiated luminal cells, which express ER, luminal markers and high levels of Gata-3, absolutely in line with our findings.

Additional interesting information about the influence of stroma on the development of preneoplastic lesions has recently been provided by a study in which we investigated the pattern of expression of the same panel of 369 genes, selected for the SH-ADH-DCIS sequence, in another type of preneoplastic lesion: hyperplastic enlarged lobular unit (HELU) [34]. HELU is a common abnormality in adult human breast and consists of an abnormal enlargement (often up to 100-fold) of the normal terminal duct lobular unit (TDLU) from which it evolves [35]. Observational studies have indicated that HELU frequently coexists with proliferative lesions more complex, including ADH and low-grade DCIS [36], or with specific breast cancer subtypes such as tubular carcinoma [37]. For this reason, HELU has been supposed to be a very early step in the biological continuum toward breast cancer [38] and it has been proposed as potential valuable risk factor to be thoroughly investigated and monitored [39], though clinical evidence has indicated that only a small proportion of HELUs actually progress to ADH, DCIS and invasive cancer [40].

Compared to normal TDLU, HELU shows only few genes differentially expressed in a statistically significant manner. In particular, in agreement with the well-differentiated lobuloalveolar phenotype, HELU underexpresses several genes typically related to the maintenance of a stem-like state (ABCG2, JAG2, NOTCH3, PROM1, SOX9 and SOX10) and overexpresses genes known to be involved in commitment to the differentiated luminal phenotype (SOX4) and mammary gland differentiation (FOXA1). Furthermore, in agreement with the essential physiological role of progesterone in mammary ductal side-branching morphogenesis and alveologenesis [41], HELU overexpresses PGR, which codes for progesterone receptor (PR). According to the classical molecular mechanism of action recognized for ligand-activated transcription factors, progesterone first binds to inactive PR, induces a conformational change leading to the dissociation of chaperon proteins from PR. Then, ligand-receptor complex moves into the nucleus where it dimerizes, binds to specific DNA sequences (progesterone response elements) within the promoter region of target genes, and recruit co-activators to facilitate communication with the basal transcription machinery. Notably, in addition to progesterone receptor, required for the canonical ligand-dependent genomic signaling, HELU constitutively expressed also progesterone receptor membrane component 1 and 2 (encoded by PGRMC1 and PGRMC2, respectively), which are able to transduce rapid non-genomic progesterone signaling in the absence of gene transcription [42]. The concomitant overexpression of genes involved both in genomic and non-genomic mechanisms of action is of particular relevance because supports the progesterone-dependence of HELU and explains the characteristic hyperplastic aspect of this lesion.

Interestingly, when we compared the panel of genes that portrayed HELU and ADH/DCIS, we found that, despite their common luminal origin, they share only few genes differentially expressed respect to the corresponding normal tissue. The finding suggests that HELU and ADH/DCIS are two distinct entities arising in a common terminal duct lobular unit in response to a different signaling from surrounding microenvironment and not sequential steps in a biological continuum toward invasive breast cancer. In particular, the terminal lobular differentiation of HELU is progesterone-dependent as indicated by the overexpression of PGR, PGRMC1 and PGRMC2. By contrast, the terminal ductal differentiation of ADH/DCIS is estrogen-dependent as indicated by the overexpression of ESR1 and GATA3 coupled with disruption of the mechanism governing the dissociation between ER expression and cell proliferation.

Collectively, these findings may have important clinical impact as they provide new insights about the possible mechanism through which ovarian hormones may drive and fuel the transformation of ADH or HELU toward an invasive tumor. In fact, it should be reminded that hyperplasia is the physiological adaptive cellular change in response to a transitory stimulus. Normally, at stimulus cessation, hyperplasia regress and the tissue reacquires a normal morphology. However, when stimulation persists or an abnormal response to the stimulus occurs, hyperplasia does not regress and cells may acquire new features that allow them to escape normal regulatory mechanisms, evolve in a frank preneoplastic lesion and ultimately in lobular or ductal invasive cancer. Our findings indicate that HELU and ADH can be the result of an abnormal and persistent presence of progesterone or estrogens that induce the constitutive expression of their own receptors with the aim to promote and sustain cell survival within the hypoxic, nutrient deprived intraductal microenvironment. It is interesting to note that increasing evidence has

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demonstrated that ovarian hormones are able to regulate positively cellular autophagy, the major cellular catabolic pathway that provides cells of an alternative source of energy in adverse conditions such as hypoxia or nutrients deprivation. In particular, in vitro studies have demonstrated that sex steroids induce autophagy in bovine mammary epithelial cells cultured in 3D-system to form acinar-like structures [43]. In addition, a recent study has demonstrated that progesterone receptor membrane component 1, involved in transduction of rapid non-genomic progesterone signaling, binds to key components of the autophagy machinery and promotes it [44].

Despite clinical evidence indicates that only a limited number of HELU and ADH evolve in invasive cancer, it is evident that the constitutive expression of sex hormone receptors might represent the first, even non-obligate, step toward neoplastic transformation, followed by the acquisition of gainful features including autocrine production of growth factors, unlimited proliferative capability, escape from apoptosis and neoangiogenesis [45]. Observation that compared to ADH, DCIS concomitantly overexpresses ESR1 and genes involved in cell proliferation (i.e., CCNB1, CCNB2 and MKI67) supports this hypothesis and provides a reasonable explanation for the clinical evidence that ovarian hormones play a key role in breast cancer development, especially in postmenopausal women, because of a hormone-replacement therapy [46] or an unbalanced ectopic production of hormones. It is well known, in fact, that parenchymal cells, mainly adipocytes and fibroblasts, are the major extra ovarian sources of estrogens due to cells capability of producing estrogenic hormones by the aromatization of androgens (e.g., testosterone, androstenedione, dehydroepiandrosterone and its sulfate) [47]. Such a local aromatization contributes to the ectopic production of estrogens that, persisting in the stroma surrounding the mammary gland, provide a long-lasting proliferative stimulus for ER-positive luminal cells [48].

Our findings suggest that the changes in cell identity associated with HELU and ADH development are likely due to alterations in the epigenetic control of gene transcription and that a possible cause for the occurring of these alterations could be an abnormal and/or persistent hormone signaling from surrounding microenvironment.

Since, unlike genetic alterations, epigenetic changes are potentially reversible, epigenome-targeting strategies, intended to revert transformed cells, could be a potentially useful strategy for cancer prevention. Reactivation of epigenetically silenced genes is achievable by treatment with DNA demethylation drugs including 5-aza-2'-deoxycytidine, or histone deacetylase inhibitors, including butyric acid, trichostatin A, valproic acid, and the recently developed entinostat and panobinostat [49, 50]. Accumulating evidence from preclinical and clinical studies indicate that, alone or in combination, DNA demethylators and histone deacetylase inhibitors can reverse epithelial to mesenchymal transition, decrease cell proliferation and survival, inhibit cell migration in vitro and eradicate established solid tumors [51, 52]. Furthermore, histone deacetylase inhibitors also proved to inhibit aromatase expression [53] suggesting their potential use as regulator in the production of estrogens from endogenous androgens.

Interestingly, recent studies have demonstrated the occurrence of a complex interplay between microRNAs and the epigenetic machinery [54]. In particular, some microRNAs have proven to regulate EZH2 expression [55, 56], and one of them (miR-26a) proved to target also ESR1 gene, providing a possible explanation for the overexpression of EZH2 and ESR1 that we observed in DCIS. In addition, recent studies have revealed that, in concert with transcription factors and histone marks, some miRNA are specifically involved in autophagy regulation [57]. Because of their emerging role in tumor biology, microRNAs have been extensively investigated with promising preliminary results in view of a potential microRNAs replacement therapy or as targets of specific oligonucleotides, called antagonim, able to silence the expression of corresponding gene [58, 59].

An exciting epigenome-targeting strategy, alternative to DNA demethylation drugs and histone deacetylase inhibitors, and based on consumption of bioactive food components, is now emerging. Preliminary but promising findings have demonstrated that several bioactive food components are capable of reverting epigenetic alterations and modify microRNAs expression and their mRNA targets [60, 61]. Therefore, it is conceivable to hypothesize that, in the next future, bioactive food components, through a personalized ‘epigenetic diet’, incorporated into one’s regular dietary regimen, might be used as an effective cancer prevention to revert the epigenetic alterations responsible for cell identity perturbation and pathological transformation.

**Conflicting interests**

The authors have declared that no competing interests exist.

**References**


