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Changed lineage composition is an early event in breast carcinogenesis

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Summary. The epithelium compartment of the human breast is made up of a branching ductal-lobular system, which is lined by a single layer of luminal epithelial cells surrounded by contractile myoepithelial cells. The co-ordinated development of these two cell types, and maintenance of their relative proportions, is fundamentally important for normal breast morphogenesis. Changes in cell type composition is one of the hallmark features of breast cancer progression, and the vast majority of breast tumors are comprised of luminal cells only, with a complete absence of myoepithelial cells. Despite this striking alteration in relative proportions of luminal and myoepithelial cells in invasive breast cancers compared with normal breast tissue, the steps in this dramatic change in cellular composition remain poorly characterised, nor is it known whether loss of myoepithelial cells is an early event in carcinogenesis. In a panel of breast tissues, we quantitated the proportion of luminal cells relative to the surrounding myoepithelial cell layer in a panel of normal and pre-invasive breast tissue samples, including lesions with proliferative disease without atypia (PDWA), columnar cell lesions (CCL), atypical ductal hyperplasia (ADH), and DCIS, and correlated these findings with proliferation in the same lesions. The study findings showed that changes in lineage composition correlate with increased proliferation, and are one of the earliest events in breast carcinogenesis. Therefore not only are myoepithelial cells important in distinguishing between invasive and non-invasive tumors, their relative proportion compared with luminal cell numbers may provide a new potential indicator of which premalignant lesions are at higher risk of progression to invasive disease.

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Introduction

The breast is a unique and dynamic organ, which displays dramatic changes in structure and function associated with each phase of development. The normal human breast is comprised of two major tissue compartments – the stroma, which consists of adipocytes, fibroblasts, blood vessels, inflammatory cells and the extracellular matrix, and the epithelium, which is made up of a branching ductal-lobular system. These ducts and lobules are lined by a single layer of luminal epithelial cells associated with secretory activity, surrounded by myoepithelial cells with contractile properties (Howard and Gusterson, 2000). Luminal cells undergo differentiation during pregnancy and synthesise and secrete milk into the lumen of the lobule during lactation, while contractile myoepithelial cells form a protective barrier between the epithelium and the surrounding stroma, and are responsible for the movement of milk out through the ducts during lactation. Studies in model systems suggest that the epithelial component in the adult breast arises from stem and progenitor cells (Visvader, 2009). These stem cells self-renew and give rise to uncommitted bipotent progenitors, which differentiate to lineage-committed progenitors and eventually to mature luminal or myoepithelial cells. The co-ordinated development of stem and progenitor cells into luminal and myoepithelial cells, and maintenance of the relative proportion of these cell types, is fundamentally important for normal breast morphogenesis.

Loss of normal tissue architecture and altered cell composition are hallmark features of breast cancer progression. It is well established that primary breast carcinomas display dramatic increases in the ratio of

luminal to myoepithelial cells, with the vast majority of breast cancers expressing only luminal markers (Abd El-Rehim et al., 2004), and that most invasive tumors lack myoepithelial cells entirely (Gusterson et al., 1982; Rudland, 1987), supporting the view that dysregulation of cell fate determination accompanies carcinogenesis. There is growing evidence that stem and progenitor cells might be targets for transformation during mammary carcinogenesis (Visvader, 2011), and the six distinct tumor subtypes identified by genomic profiling are most genetically similar to, and likely to arise from, only the stem, luminal progenitor or mature luminal cell compartments (Lim et al., 2009; Visvader, 2009).

The balance between luminal epithelial and myoepithelial cells, maintained by appropriate differentiation of epithelial progenitor populations, is important in maintaining homeostasis in the breast, and suppressing tumor formation (Barsky and Karlin, 2005; Gudjonsson et al., 2005; Polyak and Hu, 2005). Although luminal epithelial cells have been the focus of studies on the etiology of human breast cancer, myoepithelial cells also serve crucial roles in carcinogenesis, by inhibiting the proliferation of luminal cells, and contributing to tumor suppression by secreting various anti-angiogenic and anti-invasive factors (Shao et al., 1998; Nguyen et al., 2000). Therefore disruption of the myoepithelial layer results in the release of growth factors and reactive oxygen species into the tumor microenvironment, which enhances the proliferation and invasiveness of surrounding tumor cells (Polyak and Hu, 2005; Pandey et al., 2010).

In addition, the myoepithelium appears to undergo major alterations throughout carcinogenesis; the presence or absence of an intact myoepithelial cell layer is key in differentiating ductal carcinoma *in situ* (DCIS) from invasive carcinomas, and the myoepithelium in DCIS differs from normal cells in gene expression and secretes many chemokines and other factors (Allinen et al., 2004; Adriance et al., 2005). Furthermore, they play a crucial role in cell polarity through their ability to synthesise and deposit laminin-1. The loss of laminin-1 production has been shown to be the functional difference between non-malignant and tumor-derived myoepithelial cells (Gudjonsson et al., 2002). In addition, the majority of proliferation occurs within the luminal compartment whereas proliferation is exceedingly rare in the myoepithelial compartment (Joshi et al., 1986; Anderson and Clarke, 2004). It is these observations that support the hypothesis that normal myoepithelial cells function to inhibit tumor progression, and underscores the critical need to maintain a normal balance of luminal and myoepithelial cells in the breast.

Despite the striking alteration in the balance of luminal and myoepithelial cells in invasive breast cancers compared with normal breast, the steps in this dramatic change in cellular composition remain poorly characterised, nor is it known whether the altered ratio of luminal to myoepithelial cells is an early event in carcinogenesis. This study aimed to determine whether changes in lineage composition occur early in malignant transformation, whether these changes are seen in premalignant lesions that confer increased breast cancer risk, and whether altered lineage balance in the breast is accompanied by functionally important changes such as altered proliferation. We addressed this by measuring the proportion of luminal cells relative to the surrounding myoepithelial cell layer in a panel of normal and pre-invasive breast tissue samples, including lesions with proliferative disease without atypia (PDWA), columnar cell lesions (CCL), atypical ductal hyperplasia (ADH), and DCIS, and correlated these findings with proliferation in the same lesions.

Materials and Methods

Patient samples

Normal (n=24) and DCIS (n=27) breast tissue samples, and 2 tissue microarrays (TMAs; consisting of normal breast tissue, CCLs, and DCIS) were obtained from the Australian Breast Cancer Tissue Bank (http://www.abctb.org.au). Patient samples were also used from a cohort which we have previously used in another study, which included normal breast tissue, PDWA, ADH and DCIS samples (Mote et al., 2002). The use of all samples was approved by the Human Research Ethics Committee of the Sydney West Area Health Service.

Immunofluorescence histochemistry

Tissue samples and TMAs were cut into 2-μm sections and antigens retrieved as previously described (Mote et al., 2001). For immunofluorescence staining, antigens were revealed by incubation of CK18 (Sigma-Aldrich), p63 (BD Pharmingen) or Ki67 (Dakocytomation, Glostrup, Denmark) primary antibodies, followed by detection by an appropriate biotinylated secondary antibody (goat anti-mouse or anti-rabbit; Dakocytomation) and a streptavidin-conjugated fluorescent label (Alexa 594 or 488; Invitrogen, Fredrick, MD). Sections were mounted in Prolong Gold antifade reagent containing the nuclear counterstain DAPI (Invitrogen), or in Prolong Gold and counterstained with Sytox green (Invitrogen). The resulting slides were imaged with a NanoZoomer (Hamamatsu Photonics, Japan) slide scanner at 400x magnification.

Cell lineage and proliferation quantitation

Automated quantitation of the number of CK18-positive, p63-positive and Ki67-positive cells was performed using the "cell scoring" module of MetaMorph® software program (Molecular Devices). For some samples which could not be accurately quantitated automatically (due to less intense nuclear

counterstaining, for example), CK18-positive and p63-positive cells were scored using the "manually count objects" module of MetaMorph®. The number of cells counted varied depending on the size of the section or TMA core, however an average of over 4000 cells was counted per sample. For quantitation of proliferation, we subcategorized the samples into low (<1% Ki67-positive cells), medium (1-5%) and high (>5%) proliferating lesions. As approximately half of the samples contained very few detectable Ki67-positive cells, we assigned these samples as having "low", or <1%, proliferating cells. The remaining half of the cohort was split approximately equally between medium (1-5%) and high (>5%) proliferating lesions.

Statistical methods

Kruskal-Wallis non-parametric analysis of variance was used to compare the distribution of the luminal to myoepithelial ratio between lesion categories, and Spearman's rank correlation coefficient was used to examine the association of multiple parameters using SPSS software (Version 19, SPSS Inc., Chicago IL).

Results

CK18 and p63 expression in normal breast tissue and pre-invasive breast lesions

We used breast tissue samples from a number of cohorts to generate a large panel of normal and pre-invasive breast tissue samples. This panel consisted of 36 normal breast tissue samples, 8 PDWA samples, 28 CCL samples, 7 ADH samples, and 41 DCIS samples. We quantitated the luminal to myoepithelial ratio by dual immunofluorescent staining of the luminal marker, CK18, and p63, a commonly used and specific marker of myoepithelial cells in the human breast (Barbareschi et al., 2001; Reis-Filho and Schmitt, 2002; Batistatou et al., 2003), and observed variable amounts of positive expression for these markers across the different lesion types in this panel of samples (Fig. 1). Of the 120 samples analysed, 118 samples expressed both luminal and myoepithelial markers, while 2 of the DCIS samples contained only luminal cells (Table 1).

Counting of CK18-positive and p63-positive cells in normal breast tissues revealed that the median luminal to myoepithelial ratio was 1.1 (Table 2), consistent with these cell types being present in approximately equal proportions in the normal breast (Lakhani and O'Hare, 2001). Interestingly, there was an increase in the median luminal to myoepithelial ratio associated with increased lesion severity across this progression series (Table 2). Furthermore, while the lineage ratios for normal breast tissue samples showed relatively little variation, the luminal to myoepithelial ratio in other lesion categories was more widely distributed across the morphological spectrum, with the DCIS samples displaying the widest distribution range of lineage ratios (Fig. 2A).

Because of the small numbers of PDWA and ADH lesions available, we omitted these two subcategories when conducting statistical analyses in order to have sufficient power to identify statistically significant differences in lineage composition during breast cancer development. In the remaining 3 subcategories (ie. normal, CCL and DCIS), there was a highly significant difference in the luminal to myoepithelial ratio between all subcategories ($p<0.001$; Fig. 2B). These observations are consistent with an increase in the number of luminal cells, rather than a decrease in absolute myoepithelial cell numbers, being responsible for the changed lineage composition in this progression series (illustrated in Fig. 1). This is also supported by the quantitation of the average number of cells per sample across the progression series, where the absolute number of luminal cells increased markedly across the lesion spectrum, whereas the number of myoepithelial cells did not (data not shown). Therefore, we have identified a significant change in lineage composition associated with breast cancer progression, and that this change occurs early on and prior to the onset of DCIS.

Correlation between lineage composition and cellular proliferation

To determine whether there was any correlation between the lineage composition and proliferation in each lesion type, we quantitated the proportion of cells positive for the proliferation marker, Ki67, in this cohort and subcategorized the samples into low (<1% Ki67-positive cells), medium (1-5%) and high (>5%) proliferating lesions. We identified that there was a highly significant difference between the luminal to myoepithelial ratio in samples with low, medium or high levels of Ki67-positive cells ($p < 0.001$; Figure 3A). This

Table 1. Numbers and lineage composition of samples in each lesion category

	Dual lineage	Luminal only	Myoepithelial only	
Normal	36	0	0	36
PDWA	8	0	0	8
CCL	28	0	0	28
ADH	7	0	0	7
DCIS	39	2	0	41
				120

Table 2. Median luminal to myoepithelial ratio and quartile data in cohort of normal and pre-invasive breast lesions.

	n	Median ratio	25% quartile	75% quartile
Normal	36	1.1	0.8	1.6
PDWA	8	1.3	0.9	3.1
CCL	28	2.3	1.4	4.9
ADH	7	2.7	2.3	3.8
DCIS	39	8.6	4.7	14.1

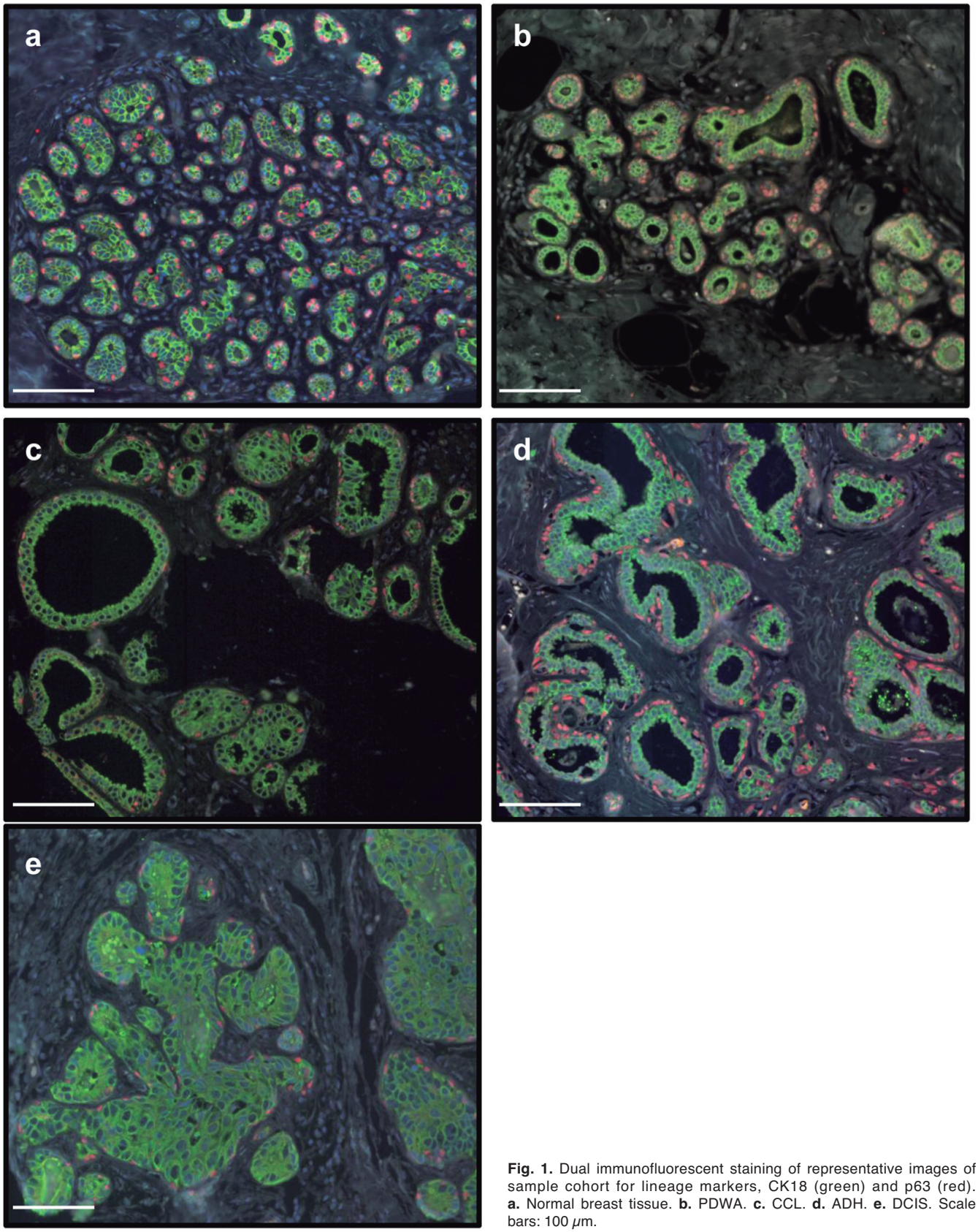

Fig. 1. Dual immunofluorescent staining of representative images of sample cohort for lineage markers, CK18 (green) and p63 (red). **a.** Normal breast tissue. **b.** PDWA. **c.** CCL. **d.** ADH. **e.** DCIS. Scale bars: 100 μm.

difference was highly significant between low and high proliferative lesions ($p<0.001$) as well as between low and medium proliferative lesions ($p<0.001$), but not between medium and high proliferative lesions ($p=0.156$). Furthermore, we observed that there is a significant correlation between lineage composition and proportion of Ki67-positive cells in these pre-invasive lesions (correlation coefficient = 0.533, $p<0.001$, Spearman's rank correlation; Fig. 3B). Finally, we observed that the proportion of proliferating cells also corresponded to the lesion subtype, with a reduction in the proportion of samples displaying low proliferative rates with increasing severity of the lesion. That is, while the majority of normal tissue displayed low proliferative rates, the majority of DCIS samples displayed high proliferative rates, as shown in Figure 4. Therefore we have demonstrated that there is a direct correlation between lineage composition, proliferation rates and severity of the lesion in pre-invasive breast lesions.

Discussion

Breast cancer is a heterogeneous disease which develops through a complex multistep process via mechanisms which are yet to be defined. The traditional hypothesis is that the majority of breast tumors arise from particular pre-existing lesions in incremental steps of increasing cellular and morphological abnormality over long periods of time (Allred et al., 2001). This is supported by multiple epidemiological studies which have identified that several common genetic, molecular and biological alterations progressively increase according to the histological subtype of premalignant disease (Santen and Mansel, 2005; Cichon et al., 2010). Furthermore, although the majority of pre-invasive breast lesions are considered non-malignant (with the exception of DCIS), they are associated with significantly higher risks for progression to invasive disease, and this relative risk also increases

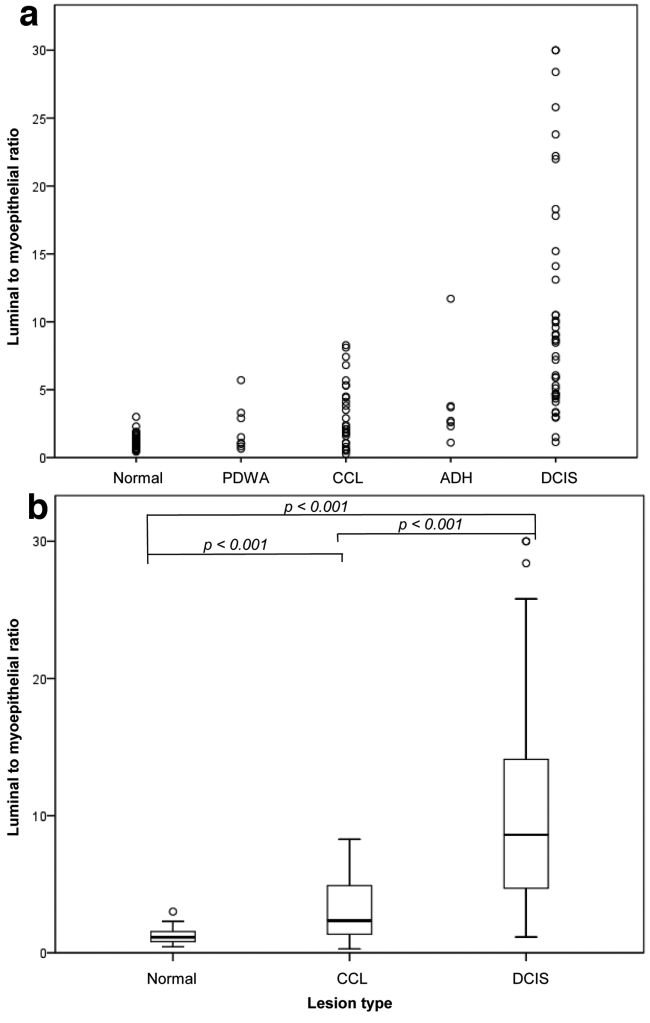

Fig. 2. a. Scatter plot of distribution of luminal to myoepithelial ratio in sample cohort. **b.** Box plot of distribution and statistical differences in lineage composition between three major subcategories.

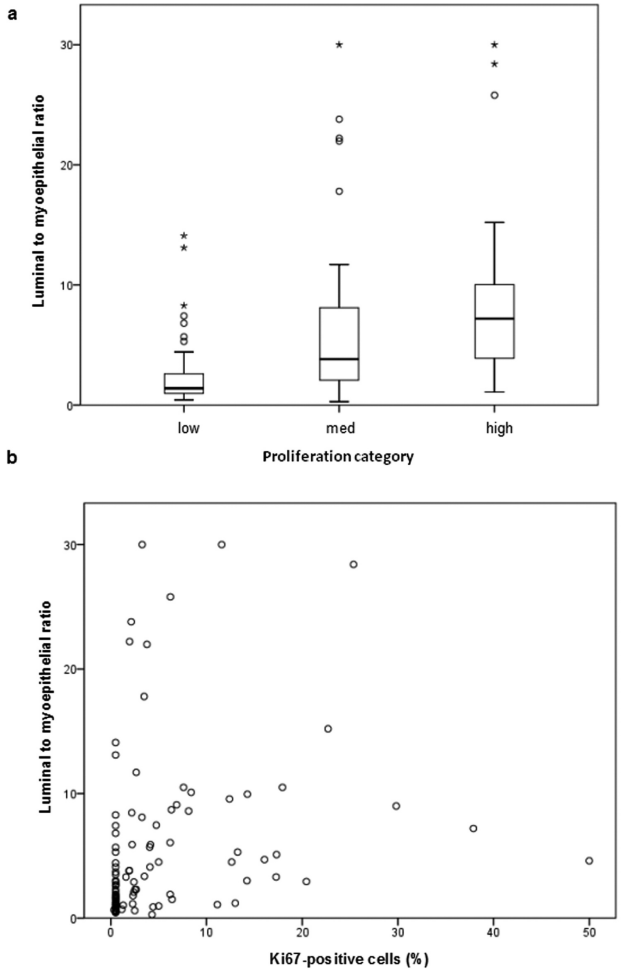

Fig. 3. a. Boxplot of distribution of luminal to myoepithelial ratio in samples with relative low, medium and high proliferation rates. **b.** Scatter plot of distribution of luminal to myoepithelial ratio and percentage of Ki67-positive cells.

progressively according to the histological subtype of the lesion. For example, while variable ranges have been reported, the relative risk factors are accepted to be around 1.3 (PDWA), 1.5 (CCL), 3.2 (ADH), and 10 (DCIS) (Arpino et al., 2005; Boulos et al., 2008; Cichon et al., 2010; Tamimi et al., 2010). However this classic model of breast cancer progression is oversimplified, as some invasive tumors arise *de novo* from morphologically normal looking cells, while only a small proportion of benign lesions actually develop into breast tumors (Newsham, 1998; Cichon et al., 2010). Therefore whether early breast lesions represent obligate precursors to invasive cancer, or whether they are merely markers of increased risk within an individual, remains unclear.

It is well-documented that the alteration and degradation of the myoepithelial cell layer occurs prior to invasive cancer, however whether this is a pre-requisite for invasion, or a bystander effect, is yet to be established. Furthermore, although the myoepithelial layer appears to be morphologically intact in DCIS, myoepithelial cells associated with DCIS harbor major molecular changes when compared to normal myoepithelial cells (Allinen et al., 2004). Here we have demonstrated that in a series of 120 normal and pre-invasive breast lesions, dysregulation of lineage specification is a very early event in breast carcinogenesis, occurring in proliferative and columnar cell lesions commonly thought not to be associated with marked increased risk of subsequent invasive cancer development, and well before the development of DCIS. Intriguingly, despite the conflicting hypotheses regarding how human breast cancer evolves, we observed an increase in the median luminal to myoepithelial ratio which corresponded with the successive steps of progression, which may reflect a progressive disruption in normal lineage development that may be functionally relevant in carcinogenesis. Moreover, we have shown a highly significant statistical difference in the distribution of lineage ratios between each of the three major lesion categories.

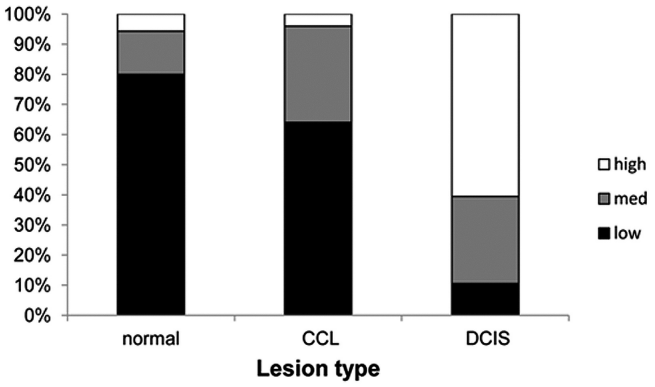

Fig. 4. Proportions of each major lesion subtype with low, medium and high proliferative rates.

We hypothesise that an increase in the number of luminal cells, rather than a decrease in absolute myoepithelial cell numbers, is responsible for the changed lineage composition in this progression series. This is supported by the quantitation of the average cell number per sample in a lesion type, and existing evidence that proliferation occurs mainly in the luminal compartment (Ferguson, 1985; Joshi et al., 1986; Perusinghe et al., 1992; Suzuki et al., 2000; Clayton et al., 2004). It has also been reported that the earliest lesions are characterised by increased number or size of glandular structures and increased number of luminal cells within the ducts and lobules (Cichon et al., 2010), without a corresponding increase in the number of cells in the surrounding myoepithelial layer of these distended ducts. However, we argue that the changes in cell lineage composition we have observed are a consequence of alterations in lineage specification, rather than merely due to an increase in proliferating luminal cells, as myoepithelial cells often disappear entirely from severe lesions and invasive tumors. In addition, we have shown that this altered lineage balance correlates with an increase in the proportion of proliferating cells, providing further support for the change in lineage composition being an increase in the number of proliferating luminal cells, rather than a reduction in myoepithelial cell numbers. The decreased relative proportion of myoepithelial cells would result in the loss of the tempering influence of these cells on the luminal epithelial compartment,

The underlying mechanisms driving the striking changes in lineage balance observed early in carcinogenesis cannot be deduced from this study. Stem cells self-renew and give rise to uncommitted bipotent progenitors, which differentiate to lineage-committed progenitors and eventually to mature luminal or myoepithelial cells (Visvader, 2009). The influences and mechanisms which drive this epithelial differentiation, and which may go awry in tumorigenesis, remain to be fully established. Interestingly, it has been shown that a subset of luminal progenitor cells is able to give rise to myoepithelial cells. It has been hypothesised that there are luminal sublineages that include ductal- and alveolar-restricted progenitors, and that the latter can commit to a myoepithelial cell fate, but that myoepithelial cells are unable to convert to the luminal lineage (Péchoux et al., 1999; Visvader, 2009). This would then support the idea that if luminal progenitor cells do not receive, or are unable to respond to some extrinsic signal that is required to trigger differentiation into myoepithelial cells, then they would continue to expand along the luminal cell lineage. Therefore, this inability to convert to the myoepithelial phenotype would explain why more than 90% of breast tumors express only luminal markers (Péchoux et al., 1999). Thus, perhaps the increase in luminal to myoepithelial ratio is due to a reduction in differentiation along the myoepithelial lineage in early breast lesions, with a subsequent increase in luminal cell numbers.

An alternative possibility is that an early event in

carcinogenesis is an increase in progenitor cells that give rise to the luminal epithelial lineage. Previous studies have shown that microenvironmental factors can dictate the cell fate decision process in multipotent progenitors (LaBarge et al., 2009), and so certain surrounding microenvironmental factors, such as changes in the hormonal milieu, or other changes consequent to the carcinogenic trigger, may potentially favour luminal differentiation of these progenitor cells. This combination of a decrease in the proportion of myoepithelial cells (and thus decreased tumor suppressor-like action), and increase in progenitor cells (and thus targets for transformation), would present a mechanism through which changes in lineage composition contribute to tumorigenesis, rather than merely serving as a marker of increased breast cancer risk. Indeed, support for this possibility has been provided very recently through the demonstration that increasing age, and therefore increased time of exposure of the breast to environmental, hormonal and other changes, is associated with an increase in the luminal to myoepithelial ratio in normal epithelial cells, as well as increased multipotent progenitor cell numbers, which the authors hypothesised to provide a cellular basis for the higher potential for malignant transformation seen in older women (Garbe et al., 2012). Similarly, perhaps this shift in lineage balance which occurs with age presents one of the earliest windows of increased susceptibility to breast lesion formation.

The wide distribution of luminal to myoepithelial ratios in the most advanced lesion, DCIS, is striking. As not all these lesions necessarily progress to invasive tumors, it would be interesting to determine whether those with a higher luminal to myoepithelial ratio indicate a higher risk of development of invasive breast cancer, and thus quantitation of the lineage ratio in these lesions may serve as an additional marker of prognostic significance. In summary, the data presented here has demonstrated that changes in lineage composition correlates with increased proliferation and is one of the earliest events in breast carcinogenesis. Therefore not only are myoepithelial cells important in distinguishing between invasive and non-invasive tumors, their relative proportion compared with luminal cell numbers may provide a new potential indicator of which premalignant lesions are at higher risk of progression to invasive disease.

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Disclosure/Conflict of Interest. The authors declare that they have no competing interests.

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