Abstract
The tumor microenvironment dynamically regulates the progression of cancer. In the breast, a unique component of the microenvironment is the myoepithelial cell. Normal myoepithelial cells act as ‘natural tumor suppressors’; however, more recent evidence suggests that these cells develop phenotypic changes, which may contribute to loss of tumor suppressor activity. We have shown that myoepithelial cells in a subset of preinvasive ductal carcinoma in situ (DCIS) upregulate expression of the integrin αvβ6, switching on tumor promoter activity through activation of TGFβ and MMP9. This makes the tumor microenvironment more permissive to invasion, seen both in vitro and in vivo. In human tissue samples, increased myoepithelial αvβ6 expression correlated with increased risk of disease progression and recurrence. Current estimates suggest that as many as 50% of DCIS cases will never progress in the patient’s lifetime, but there are no markers to predict the outcome of individual cases. The identification of αvβ6 in a subset of DCIS presents a unique way to stratify patients with DCIS into those who may or may not progress to more serious disease. As αvβ6 is not expressed on most normal adult tissues, this finding may also provide novel targets for therapy in this high-risk group. Cancer Res; 74(21); 5942-7. ©2014 AACR.

Introduction
There has been growing concern that breast cancer screening may result in overdiagnosis and overtreatment of the disease (1). In the United States, around 60,000 cases of preinvasive ductal carcinoma in situ (DCIS) are diagnosed each year, accounting for 1 in 5 new breast cancer cases (American Cancer Society 2014, www.cancer.org/cancer/breastcancer/detailedguide/breast-cancer-breast-cancer-types), for which the natural history remains uncertain.

In DCIS, neoplastic epithelial cells proliferate and fill the lumen of the breast duct, but these tumor cells remain separated from surrounding stroma by the intact myoepithelial-basement membrane barrier. When DCIS progresses, tumor cells penetrate the myoepithelial-basement membrane and move into the interstitial matrix to become an invasive carcinoma with metastatic potential, and ultimately the myoepithelial cells are lost.

Since the advent of screening, DCIS has been more frequently diagnosed; however, studies have shown that around 50% of diagnosed DCIS will not progress to invasive disease during a woman’s lifetime (2, 3). The current standard of care for DCIS is surgical excision, with mastectomy if the DCIS is extensive, which has a risk of recurrence of 1% to 2% or, for more limited disease, lumpectomy with adjuvant radiotherapy, which carries a risk of recurrence of 12%. Regardless of the treatment, less than 2% to 3% of these patients will die from breast cancer (4, 5), and there is concern that for many women, current management represents significant overtreatment. The NIH State-of-Science conference on DCIS recently highlighted the need to understand more about the biologic and molecular mechanisms underlying the progression of DCIS to invasive disease to achieve robust prognostic and therapeutic patient stratification (2). In 2010, Monica Morrow, Chief of Breast Services at Memorial Sloan-Kettering (New York, NY), stated that a ‘molecular marker that could predict which DCIS would progress to invasive cancer versus those that would forever stay DCIS would be an enormous clinical advance’ (5), highlighting the need for robust predictive biomarkers for this disease.

Prognostic Markers in DCIS
The mainstay of DCIS classification is morphology, based predominantly on nuclear grade and architectural features. Other factors correlating with risk of recurrence or progression include younger age at diagnosis, larger sized tumors, and positive margins (i.e., the cancer being incompletely excised at surgery; ref. 5).

The Van Nuys Prognostic Index for DCIS was developed to predict recurrence, and includes five characteristics: size of the tumor, margin width, presence of necrosis, nuclear grade, and age of the patient. DCIS cases receive a score of between 4 and 12, 4 being the least likely to recur and characterized as an older
women with well excised, small low-grade lesions. Those with a score of 12 are most likely to recur, and are characterized as a younger woman with a large, incompletely excised, high-grade tumor. However, the index is rarely used in the manner for which it was designed, as it is not able to predict which individual case will recur with invasive cancer or DCIS (6).

Several studies have attempted to identify markers or gene signatures that can predict progression of DCIS. The majority of these studies have tried to identify differences between DCIS tumor cells and their invasive component. Ma and colleagues (7) compared 36 cases comprising normal breast tissue, atypical ductal hyperplasia (ADH), DCIS, and invasive ductal carcinoma (IDC), which represent the traditional step-wise progression of breast cancer. These cases would be expected to show increasing genetic abnormalities as they progress from normal to IDC and the aim was to identify genetic markers that would pinpoint the stages of breast cancer evolution. Surprisingly, gene expression analysis demonstrated very few differences between the different stages, with the greatest number of genetic alterations exhibited at the ADH stage and maintained through DCIS and IDC, suggesting that the gene expression profile of early-stage disease largely reflects that of advanced cancer. This work has since been corroborated by others; for example, Castro and colleagues (8) analyzed 22 invasive breast cancers with concomitant DCIS as well as five cases of pure DCIS and 10 cases of IDC. They found that the tumor cells with the most divergent molecular features were from the pure DCIS cases and concluded that genetic alterations occur before the morphologic changes associated with invasive breast cancer. A candidate gene approach taken by Moolans and colleagues (9) analyzed copy number of 21 cancer-associated genes (including FGFR1, EGFR, HER2, MYC) in 39 cases of DCIS and IDC, and similarly found no significant differences between the two groups, though they identified differences in the number and pattern of the alterations between low- and high-grade DCIS (9). A study carried out by Kerlikowske and colleagues (10) examined established breast cancer biomarkers; ER, PR, p53, ERBB2, p16, COX-2, and Ki-67 in a cohort of 1,162 DCIS cases, in which 324 patients developed a subsequent invasive disease. A study by Solin and colleagues (11) carried out by Kerlikowske and colleagues (10) examined established breast cancer biomarkers; ER, PR, p53, ERBB2, p16, COX-2, and Ki-67 in a cohort of 1,162 DCIS cases, in which 324 patients developed a subsequent invasive disease. In a multivariate analysis, they found that DCIS lesions detected by palpation or those positive for p16, COX-2, and Ki-67 were significantly associated with subsequent invasive cancer. The authors go on to suggest that high Ki-67 indicates a high proliferative index, p16 is a validated marker of basal–phenotype (which often are aggressively invasive), and COX-2 has an established role in promoting invasive potential. Therefore, these three markers may provide a biologic rationale for why this subset of DCIS is more likely to recur. More recently, a modified form of the Oncotype DX recurrence score for invasive breast cancer has been developed for DCIS. The original recurrence score uses 16 cancer genes and five reference genes, whereas the Oncotype DX DCIS score is modified to use seven cancer genes, five of which measure proliferation (Ki-67, STK15, survivin, CCNB1, MYB2, PR, and GSTM1), with five reference genes to quantify local recurrence risk. A study by Solin and colleagues tested this Oncotype DX DCIS assay on 327 patients with DCIS and demonstrated a 10-year risk for three prespecified DCIS risk groups of 10.6% (low), 26.7% (intermediate), and 25.9% (high). The DCIS score is limited by the paucity of DCIS samples from which predictive genes and algorithms could be identified; therefore, invasive breast cancer cases had also to be used. However, the difference in risk of developing local recurrence and invasive local recurrence between patients with lower DCIS scores and higher DCIS scores is statistically significant (11).

In recent years, the biologic heterogeneity of invasive breast cancer has been reflected in a molecular classification of tumors, placing cancers into "intrinsic" subtypes associated with clinical outcome (12). This heterogeneity is already present in DCIS, though with some differences in the relative frequency of subtypes between DCIS and invasive carcinoma (13), with a greater proportion of DCIS cases exhibiting HER2+ and PR− expression (14.9%) compared with invasive carcinoma (3%–6%) and lower proportion of DCIS cases characterized as triple-negative/basal cancers (11.7%) compared with invasive carcinoma (11%–20%). However, it is clear that progression occurs in all subtypes and this intrinsic classification does not distinguish between those more or less likely to develop invasive disease.

DCIS and the Microenvironment

The apparent lack of genetic evolution between DCIS and invasive breast cancer has focused attention on the potential role of the microenvironment in mediating the transition to invasion. The potential importance of the tumor microenvironment has been known since 1863 when Virchow observed leukocytes in the stroma of neoplastic tissue. The modern paradigm for the role of the tumor microenvironment in breast cancer was set down by Bissell and colleagues (14), who postulated that the microenvironment in which a tumorogenic cell evolves is as critical to its evolution as the genetic mutations that it accrues. This was proven in a set of experiments carried out by Dolberg and Bissell (15) using Rous sarcoma virus (RSV). RSV contains a prototypical oncogene v-src and injection of RSV into the web wing of newly hatch chicks induced sarcomatous tumors. However, when Dolberg and Bissell injected the virus into 4-day-old embryos, they found the transforming function of the virus to be overridden by the microenvironmental context and no tumors developed, reviewed in ref. 16.

There is emerging evidence that the microenvironment may also play a role in DCIS. In a gene expression array analysis, Muggurud and colleagues (17) identified a distinct subset of DCIS, independent of grade or hormone receptor status, which clustered with invasive carcinomas. These cases were characterized by high proliferation and enriched for genes associated with stromal reorganization, including matrix metalloproteinases (MMP) and extracellular matrix (ECM) proteins, suggesting that creation of a microenvironment permisive to invasion may be a key step in DCIS progression. Further emphasizing the role of the microenvironment, Sharma and colleagues (18) identified two distinct stromal signatures initially in invasive carcinoma (but also in DCIS). These
signatures derived, respectively, from a macrophage response and a fibroblastic response. They demonstrated that those cases with a macrophage response signature had a more aggressive phenotype, suggesting the microenvironment influences disease behavior. The Lisanti group has worked extensively on the role of the microenvironment as a predictor of DCIS progression and recurrence in recent years. In 2009, Witkiewicz and colleagues showed that expression of caveolin-1 (Cav-1) in breast fibroblasts surrounding affected ducts was strongly linked to progression. Nearly 99% (7/8) of the patients with DCIS that had recurrence to invasive breast cancer had reduced or absent levels of Cav-1 in the stroma, whereas 97% (35/36) of ER "high" tumors did not show invasive recurrence over the follow up period (4–208 months; ref. 19). A further role for the stroma influencing disease behavior was shown in a study of 97 DCIS cases where stromal expression of CD10 or SPARC associated strongly with time to recurrence and recurrence status; together CD10 and SPARC strongly correlated with shortest time to recurrence (20).

The importance of the stroma is also demonstrated in animal models. Enhanced collagen cross-linking mediated by stromal-derived Lysyl Oxidase (LOX) led to the promotion of growth and invasion by premalignant Ha-ras MCF10AT mammary epithelial cells injected into mouse mammary fat pads. Furthermore, in vitro 3D collagen gel culture systems directly demonstrated that increased "stiffness" of the ECM could drive invasion of oncogenically initiated mammary epithelial cells in a mechanism dependent on increased β1-integrin activity (21). These studies demonstrate the impact of the peri-ductal microenvironment on epithelial cell behavior though focusing primarily on the extracellular matrix proteins.

The Role of the Myoepithelial Cell in DCIS

The microenvironment of DCIS is unique in comprising a layer of myoepithelial (also called "basal") cells that separate the neoplastic epithelial cells from the stromal compartment. Thus, myoepithelial cells provide the interface between tumor cells of DCIS and the microenvironment, providing stable anchorage to the basement membrane via hemidesmosome formation. This cell layer could be regarded merely as a physical barrier, but there is growing evidence of a regulatory role played by myoepithelial cells in the mammary gland. Normal myoepithelial cells have been shown to exhibit anti-angiogenic (22), anti-proliferative (23), and anti-invasive (24, 25) properties, as well as express a variety of recognized tumor suppressor proteins (p63, p73, 14-3-3-σ, maspin; refs. 26–28). They have been shown to induce polarity in luminal epithelial cells through cell-type–specific desmosomal proteins (29), and through release of laminin-1 (25). However, several observations suggest that the myoepithelial phenotype is altered in DCIS (Fig. 1). In DCIS ducts, the cells show loss of hemidesmosome formation (30) and a switch in their secretion of ECM isoforms toward a more fetal phenotype that promotes cell migration (31). Furthermore, myoepithelial cells from DCIS have been shown to lose the ability to polarize luminal cells and lay down basement membrane (especially laminin-1; ref. 32). Allen and colleagues (33) showed that myoepithelial cells exhibit more dramatic gene expression changes than any other cell component between normal and DCIS tissues, suggesting extensive abnormal paracrine interactions in DCIS. Indeed, xenograft studies suggest that dedifferentiation of host myoepithelial cells leads to the transition of in situ to invasive disease. Together, these studies suggest that DCIS-associated myoepithelial cells lose their tumor suppressor function and may actually promote breast cancer progression, though this has not previously been confirmed in functional systems.

In our recent study (34), we examined the expression of αβ6-integrin in 583 breast samples comprising: 28 reduction mammoplastys, 23 cases with epithelial hyperplasia of usual type, 238 pure DCIS cases, and 294 DCIS cases with concomitant invasion. Myoepithelial cells showed no staining for αβ6-integrin in normal or hyperplastic tissue, but 69% of high-grade and 52% of non–high-grade pure DCIS contained myoepithelial cells positive for αβ6. In DCIS with associated invasion, 96% of the high-grade cases were found to have myoepithelial cells positive for αβ6.

The integrin αβ6 is restricted to epithelial cells and is not normally expressed in adult human tissue except during wound healing. The primary ligand of αβ6 is the RGD-containing motif of the latency-associated peptide (LAP) of latent-TGFβ (types I and III; ref. 35) and upon ligation to LAP, αβ6 causes TGFβ to become activated. One of the downstream consequences of this is the activation of certain MMPs (e.g., MMP3 and MMP9; refs. 36, 37). Activation of MMPs plays a central role in remodeling the ECM and changes in the activation patterns of MMPs in cancer is thought to promote basement membrane degradation and tumor invasion (38). Previous studies have identified αβ6 expression in a number of invasive carcinomas and the current dogma suggests that expression of this integrin is associated with aggressive tumor behavior and reduced survival (39–41).

Our study is the first to identify upregulation of αβ6 in a nontumorigenic microenvironment cell population. Analysis of a spectrum of lesions, from normal through hyperplasia, pure DCIS to DCIS that have progressed to invasive disease suggested that myoepithelial expression of αβ6 increases with the acquisition of more aggressive tumor features, and may even promote the transition from in situ to invasive disease.

To further investigate the association between myoepithelial expression of αβ6 and disease progression, we analyzed tissue samples from patients entered into the UK/ANZ DCIS trial with associated long-term follow-up (median 114 months). αβ6 expression was assessed in 52 case–control pairs of DCIS cases for an association with any new breast cancer (DCIS or invasive). We found a significant correlation between myoepithelial αβ6 expression and disease recurrence or progression (P = 0.006), which remained significant after adjusting for DCIS size and grade (P = 0.02). DCIS cases positive for αβ6 also demonstrated a much shorter median recurrence/progression time than those without αβ6, at 2.3 years versus 11.4 years, respectively.

To address more directly the functional significance of myoepithelial-associated αβ6, we created a model of
DCIS-associated myoepithelial cells derived from a normal myoepithelial cell line (kindly provided by Prof. Mike O’Hare, Emeritus Professor at University College of London, London, United Kingdom) manipulated to overexpress αvβ6. Using this model conditioned media from αvβ6-positive myoepithelial cells led to increased invasion by tumor cells derived from a variety of tumor molecular subtypes. This effect on tumor cell invasion could be recapitulated using primary myoepithelial cells isolated from normal breast tissue, but only if they were transiently transfected with αvβ6. Using zymography and blocking antibodies, we demonstrated that myoepithelial cells expressing αvβ6 promoted a TGFβ-dependent upregulation of MMP9 and that blocking MMP9 in Transwell invasion assays ablated the enhanced tumor cell invasion induced by αvβ6 expression in myoepithelial cells.

When MDA-MB-231 breast cancer cells were coinjected with either αvβ6-positive or -negative myoepithelial cells into the mammary fat pads of SCID mice, an increased tumor cell growth was evident in the presence of αvβ6-positive myoepithelial cells. Relating back to DCIS tissue samples, we could clearly demonstrate a significant association between αvβ6 expression in the myoepithelial compartment and MMP9 expression in the same region of serial sections (P < 0.0001).

Taken together, this indicates that αvβ6 is a robust marker in DCIS-associated myoepithelial cells, which leads to altered myoepithelial behavior, switching them from tumor suppressors to tumor promoters. Our data suggest that αvβ6 expression in the myoepithelial cells of DCIS is a marker of poor prognosis and identifies those patients at high risk of progression or recurrence.

Work from Professor Weaver’s group has demonstrated that cancer tends to arise in regions of increased tissue stiffness (21). Her group has also shown that as tissue stiffness increases so does the risk of invasion and metastasis (42). Recently, it has been demonstrated that myoepithelial cells are able to sense and respond to increased matrix stiffness via α5β1 binding to fibronectin, thus restoring tensional homeostasis and reducing the forces, the cells experience to normal levels. This response is eventually overcome as the tissue stiffens; however, when myoepithelial cells express αvβ6, their ability to respond to a stiffening microenvironment is lost and consequently the cells experience the increase in matrix stiffness more immediately (43). These observations suggest another mechanism by which αvβ6 ablates the natural tumor suppressor response of myoepithelial cells and could lead to faster progression of DCIS to invasive disease.
Future Directions

Our findings provide mechanistic data of how changes in the microenvironment of early breast cancer lesions can determine tumor behavior, supporting the observations of others (16). Our data show for the first time how a change in the nonmalignant population of myoepithelial cells surrounding the tumor mass is critical to the switch from a tumor suppressive to a tumor permissive and even tumor promoting microenvironment. Upregulation of αvβ6 driving an increase in invasive capacity and stromal reorganization is consistent with its function in other cancers, but the key difference here is that αvβ6 is able exert this effect via a nontumorigenic supporting cell type.

It is clear that further studies are needed to establish whether αvβ6 expression in the myoepithelial cell compartment of DCIS can be used in a clinical setting to stratify patient care. Other components of the microenvironment, including stroma and vasculature, are also likely to be important in facilitating invasion. Studies also have shown that characteristics of the tumor cells need to be taken into account, in particular, the proliferative activity of the lesion, as demonstrated by the Oncotype DX Score and Kerlikowske and colleagues (10) to enhance prognostic power. Other markers such as p16, COX-2, and Ki-67 as used by Kerlikowske and colleagues (10) to enhance prognostic power. This is highly plausible because our laboratory has previously shown a strong positive correlation between αvβ6 and COX-2 expression in oral squamous cell carcinoma (OSCC; ref. 44). This leads to the exciting possibility of new therapeutic avenues in the treatment of DCIS as we have shown that NSAIDs are able to downregulate both COX-2 and αvβ6 in OSCC (44). There may be other pathways that lend themselves to targeting in this model, such as TGFβ or MMP9, which have not been tested in early breast cancer. This may represent a key point in the evolution of breast cancer that could be used both predictively and prognostically in the tailored management of women with DCIS.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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αvβ6 Expression in Myoepithelial Cells: A Novel Marker for Predicting DCIS Progression with Therapeutic Potential

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