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What is the malignant nature of human ductal carcinoma *in situ*?

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Abstract

Invasive, genetically abnormal carcinoma progenitor cells have been propagated from human and mouse breast ductal carcinoma *in situ* (DCIS) lesions, providing new insights into breast cancer progression. The survival of DCIS cells in the hypoxic, nutrient-deprived intraductal niche could promote genetic instability and the derepression of the invasive phenotype. Understanding potential survival mechanisms, such as autophagy, that might be functioning in DCIS lesions provides strategies for arresting invasion at the pre-malignant stage. A new, open trial of neoadjuvant therapy for patients with DCIS constitutes a model for testing investigational agents that target malignant progenitor cells in the intraductal niche.

Research on the pre-malignant stages of breast cancer is beginning to address a fundamental question in human cancer biology: when does the invasive phenotype first arise? Recommendations from the 2009 US National Institutes of Health (NIH) breast ductal carcinoma *in situ* (DCIS) consensus conference[1] have highlighted the clinical controversies that surround the treatment of DCIS (also known as intraductal carcinoma) and the need to understand the malignant nature of DCIS. Although some members of the NIH DCIS conference proposed that the word ‘carcinoma’ should be removed from the term DCIS, because DCIS is non-invasive and has a favourable prognosis, experimental studies of human and mouse DCIS lesions are showing the opposite: carcinoma precursor cells exist in these lesions, and the aggressive phenotype of breast cancer is predetermined early at the pre-malignant stage[2–8]. Investigators are uncovering the mechanisms through which DCIS and other pre-cancerous cells survive and adapt in the stressful, hypoxic, nutrient-deprived intraductal microenvironment[4,9–12]. Cyto-genetically abnormal DCIS progenitor cells have been isolated and propagated from fresh human DCIS lesions[4] and mouse DCIS models[3,7]. Understanding the cellular processes that promote the survival of malignant progenitor cells in DCIS is providing strategies for killing DCIS cells.

In this Opinion article we highlight how the high-stress environment of the breast intraductal niche could spawn genetically abnormal malignant progenitor cells. On the basis of insights derived from studies of propagated spheroid-forming cells that were cultured from fresh DCIS lesions, a novel neoadjuvant therapy trial has been opened for patients with DCIS. The special design of this trial offers a means to screen investigational agents that suppress or kill malignant progenitor cells in pre-invasive breast lesions.

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Competing interests statement
The authors declare no competing financial interests.

FURTHER INFORMATION
ClinicalTrials.gov: http://clinicaltrials.gov/ct2/home
NIH Consensus Development Program: http://consensus.nih.gov/2009/dcisstatement.htm
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DCIS

DCIS is the most common type of non-invasive breast cancer in women[13]. The incidence of DCIS has increased sevenfold from the mid-1970s, primarily owing to increased detection through the widespread adoption of radiographic screening for invasive carcinoma. According to the 2009 NIH DCIS consensus conference, the incidence rate for women aged 50–64 years is 88 per 100,000 (REF. 1), and as of 1 January 2005 an estimated 500,000 women were living with a diagnosis of DCIS.

DCIS is defined as a proliferation of neoplastic epithelial cells within the closed environment of the duct, which is normally surrounded by myoepithelial cells and an intact basement membrane[13–15]. The outside perimeter of the basement membrane interfaces with the connective tissue stroma, immune cells, lymphatics and vasculature[13] (FIG. 1). By definition, DCIS has not yet invaded beyond its intraductal origin, and might never invade neighbouring tissues. However, there is both clinical and experimental evidence to suggest that DCIS is a precursor lesion to most, if not all, invasive breast carcinomas[8,13,16–18]. It is generally accepted that women diagnosed with DCIS remain at high risk for the subsequent development of invasive carcinoma[19–21]. However, the proportion of DCIS lesions that would progress to invasive breast cancer if left untreated is unknown. Lesion size, degree of nuclear atypia and the presence of comedo necrosis are histopathological parameters that have been identified as affecting the risk of recurrence in the heterogeneous range of pre-malignant breast lesions[13,22,23]. DCIS is classified — based on the level of pathological characteristics and cytological abnormalities — into high-grade, intermediate-grade and low-grade lesions[8,13,15], including the presence or absence of central necrosis[18,24]. The most aggressive type of DCIS is comedo-DCIS, which is frequently associated with central necrosis and micro-calcifications (small deposits of hydroxyapatite) and a high cytological grade[13]. Additional morphological forms of DCIS include cribriform (open spaces between the cords of cells), papillary or micropapillary (having finger-like projections) and solid. Many DCIS cases include at least two different architectural and molecular subtypes in the same breast[8,13,15,16,18,22]. Although there are many unanswered questions relating to the progression of DCIS to invasive breast cancer (BOX 1), the capacity of DCIS cells to survive in the hypoxic and nutrient-deprived environment of the intraductal niche is perhaps a crucial step towards malignancy.

Box 1

DCIS unanswered biological questions

Although the transition from *in situ* to invasive cancer is central to the origin of the malignant phenotype, little is known about the time of onset or the triggering mechanism that switches *in situ* neoplastic lesions to overt invasive carcinoma in the human breast. Finding the answers to the questions listed below should help us to understand more about the timing and onset of malignant breast cancer.

- Why do some ductal carcinoma *in situ* (DCIS) lesions progress to stromal invasion, but others apparently lie dormant? Are the subsets of DCIS lesions destined to progress fundamentally differently from those with a benign outcome?
- Alternatively, do neoplastic cells with invasive potential arise frequently within DCIS lesions, but are held in check in lesions with a dormant clinical course?
- DCIS cells that accumulate in the non-vascular intraductal space are under severe hypoxic and metabolic stress. Which mutations are selected for that

promote the survival of DCIS cells to this stress? Are such survival pathways targets for chemoprevention or intervention?

- Does adaptation to stress within the duct contribute to the carcinogenic process[12,25,27]?
- The genetic abnormalities found in high-grade DCIS lesions are largely identical to the genetic abnormalities identified in the matched invasive carcinoma in the same patient[2,5,6,13]. Does this mean that the malignant genotype of breast cancer is predetermined at the pre-malignant stage? If so, what triggers the invasive cells to emerge?
- If potentially invasive, cytogenetically abnormal neoplastic cells pre-exist in the intraductal DCIS lesion before the overt histological transition to invasive carcinoma, are these always the ultimate source of invasive metastatic carcinoma?

Survival in the intraductal niche

Survival and adaptation of pre-malignant cells within the stressful hypoxic and nutrient-deprived intraductal microenvironment might promote genetic instability and the selection of neoplastic cells with invasive potential. Indeed, metabolic and hypoxic stress within the tumour microenvironment is known to induce mutagenesis and genetic instability[11,12,25–30]. Adaptation to survival under stress can override normal cellular stress responses, leading to the persistence of genetically damaged cells and carcinogenesis[11,12,27,29]. Genotoxic, metabolic, hypoxic and oxidative stress engage stress-response programmes in normal cells[10,12,22,29] (FIG. 2). For example, if DNA damage resulting from a genotoxic environment is substantial then such cells are likely to senesce or undergo programmed cell death. Pre-malignant and malignant cells have been proposed to either downregulate proteins that induce these pathways or upregulate pro-survival pathways[9–12,22,26,28,29,31]. Nevertheless, even if a cell can resist programmed cell death or senescence it will still not survive in a hypoxic, nutrient-deprived environment unless it can find alternative sources of energy for cellular functions, such as through autophagy, anaerobic respiration or increasing the efficiency of aerobic respiration[9–11,22,25,26,28,32,33]. To appreciate how DCIS might progress and circumvent stress-induced death or senescence, and use alternative sources of energy, we need to consider the nature of the stresses that affect DCIS cells.

DCIS cells must adapt to hypoxic stress within the duct

The vascular density of tissues is homeostatically regulated in all metazoan species to restrict the maximum distance between tissue cells and the nearest blood vessel. The average distance between vessels in tissues falls in the range of 25 to 50 micrometres[34,35]. The nearest distance from any DCIS cell to the vasculature on the other side of the basement membrane is determined by the DCIS lesion radius. The radii of DCIS lesions can be as great as 200 to 500 micrometres (15 to 60 cell diameters)[13]. This greatly exceeds the homeostatic limitations of blood vessel minimum density. Consequently, DCIS cells must adapt to severe hypoxic stress within the duct[28,29,36–38], thereby promoting carcinogenesis[11,12,39,40] (FIGS 1c,2b).

Multi-cellular spheroids that are grown in culture provide a model for the limitations of oxygen diffusion in a non-vascularized DCIS colony packed within the duct boundary of the basement membrane. Spheroids grown in culture exhibit central necrosis when the radius of the spheroid exceeds the maximum distance required for oxygen to diffuse in from the surface of the spheroid. The width of the viable rim of multi-cellular tumour spheroids that

are grown in spinner culture was reported to be 150 micrometres over a wide range of spheroid diameters from 400 to 1,000 micrometres[36,41,42]. This distance is in the range of the average distance of vessels from the nearest area of necrosis that has been studied in solid tumours[43]. DCIS lesions normally expand to a diameter greatly exceeding the limitations of oxygen diffusion. Comedo-DCIS lesions show a central zone of necrosis with a sharp border of surrounding viable, neoplastic cells (FIGS 1,2). The viable rim can range from 5 to 25 cell layers thick[24,37,38]. Morphometric studies have shown that the mean diameter of the ducts containing DCIS with necrosis is 470 micrometres, compared with a mean diameter of 192 micrometres for DCIS without necrosis[37]. Necrosis is found in 94% of ducts larger than 180 micrometres in size compared with 34% of ducts less than 180 micrometres in size[24]. These findings support the existence of a hypoxic compartment in DCIS. The specific trigger of necrosis is unknown, but insufficient ATP production to maintain plasma membrane integrity could result in metabolic catastrophe[44], which generates the typical comedo-DCIS central zone of cell lysis (FIGS 1,2). Dying cells produce soluble or endogenous factors that induce autophagy in surviving cells[45]. As discussed below, autophagy is one probable mechanism by which cells find an alternative source of ATP to avoid metabolic catastrophe[11,28,46].

Avoiding hypoxia-induced apoptotic death

In hypoxic conditions, hypoxia-inducible factors (HIFs) mediate the adaptive response to maintain oxygen homeostasis[12,47]. Under normal oxygen levels prolyl hydroxylases (PHD1–PHD3) use oxygen as a substrate to modify proline residues on the oxygen-dependent subunit HIFα. Hydroxylated HIFα is recognized by the von Hippel–Lindau (VHL) tumour suppressor protein, which is part of an ubiquitin ligase complex, and thereby targeted for proteasomal degradation[48]. Consequently, normal oxygen levels are associated with the degradation of HIFα, and, conversely, HIFα levels are increased by hypoxia. Increased HIFα levels correlate with hypoxia in solid tumours, which is associated with increased rates of patient mortality and treatment failure[49]. This, in part, might be due to the effect of hypoxia on the DNA-damage response. HIF-mediated adaptation to hypoxia can inhibit p53-mediated cell death in cells with DNA damage[12]. Moreover, hypoxic stress, independently of HIF, is associated with a decreased rate of repair of DNA damage, and increased cell invasion and metastatic potential[25,27,39,40,50,51]. Thus, in the presence of hypoxic stress, we can postulate that proliferating intraductal DCIS epithelial cells may adapt to survive in the presence of genetic mutations that drive tumour progression and facilitate invasion.

Avoiding stress-induced senescence

Abrogation of the retinoblastoma (RB) tumour suppressor pathway in DCIS cells can be an important survival strategy[22]. The dephosphorylation of RB in response to oxidative or metabolic stress in normal cells drives cell cycle arrest and senescence. It has been proposed that a subset of DCIS cells use various mechanisms to directly or indirectly compromise RB function[10,17]. One product of the *CDKN2A* locus, INK4A, is a cyclin-dependent kinase inhibitor (CDKI) that is a negative regulator of D-type cyclins. Expression of INK4A inhibits RB phosphorylation and progression through the cell cycle. INK4A also functions in preventing centrosome dysfunction and genomic instability[52]. DCIS that exhibits high INK4A immunostaining and increased Ki67 expression (high proliferation index) identifies women who have reduced recurrence-free survival. Conversely, DCIS exhibiting high INK4A immunostaining in the absence of proliferation identifies women who have a low probability of subsequent disease[10,22]. Paradoxically, overexpression of INK4A can be associated with opposite biological responses. A cell with functional INK4A–RB signalling will initiate a stress-induced overexpression of INK4A resulting in a proliferative arrest that is characteristic of cellular senescence. By contrast, a cell with a compromised RB pathway

will initiate a regulatory-induced overexpression of INK4A owing to unblocked negative feedback. In this situation the cells will disregard stress signals and will continue to proliferate[10,22].

The repression of INK4A activity, accompanied by the inactivation of RB as a transcriptional repressor, leads to the overexpression of chromatin-remodelling proteins such as the polycomb proteins enhancer of zeste homologue 2 (EZH2) and suppressor of zeste 12 homologue (SUZ12)[53,54]. These proteins are expressed in approximately 50% of DCIS lesions and are indicative of a poor prognosis in patients with invasive breast cancer[55,56]. Abnormalities in chromatin remodelling are thought to have a role in nuclear atypia and abnormalities in cell polarity, which are prominent features of DCIS pathology. In addition, when mammary epithelial cells were exposed to a variety of stresses, the inhibition of RB resulted in the persistence of DNA damage and could drive the expression of the pro-inflammatory protein cyclooxygenase 2 (COX2)[9,57,58]. COX2 expression can stimulate cell migration and angiogenesis, and has been reported to be upregulated in DCIS lesions in women with a high likelihood of subsequent breast cancer[10,58]. Therefore, another potential consequence of adaptation to stress in the intraductal niche is the promotion of genetic and epigenetic dysfunction through the INK4A–RB pathway.

Genetically abnormal DCIS cells

Breast cancer progression is thought to be a multistep process involving a continuum of changes from normal phenotype through hyperplastic lesions, carcinoma *in situ* and invasive carcinoma, to metastatic disease[8,59] (BOX 2). In this model, additional genetic alterations are required before cells in a DCIS lesion can progress to an invasive and metastatic carcinoma. We propose that the traditional stepwise model of breast cancer progression has to be revised on the basis of new knowledge about cancer progenitor cells and cancer stem cells[60]. Recent data indicate that the aggressive phenotype of breast cancer is determined at the pre-malignant stage, much earlier than previously thought. Experimental approaches employing loss-of-heterozygosity (LOH), and comparative genomic hybridization (CGH), as well as mutational screens, reveal that most of the genetic changes that underpin invasive breast cancer are evident in DCIS lesions[2,3,5–8,59,61,62]. Gene expression studies of patient-matched tissues, including atypical ductal hyperplasia (ADH), DCIS and invasive carcinoma, revealed that the various stages of disease progression are similar to each other at the level of the transcriptome[2,5,6]. These studies also show that DCIS lesions are more similar to the invasive breast cancer in the same patient compared with DCIS lesions from other patients[2,5,6]. Further support for the conclusion that primary genetic changes are present at the pre-malignant stage comes from studies of *PIK3CA* mutations and *ERBB2* (also known as *HER2*) amplifications in DCIS lesions and matched invasive lesions from the same patient[62]. No significant difference in the frequency of *PIK3CA* mutations was found[62] between the DCIS and the matched invasive cancer. In a separate study[61], no significant differences were found between the gene amplification status of *ERBB2*, oestrogen receptor 1 (*ESR1*), cyclin D1 (*CCND1*) and *MYC* in DCIS compared with invasive breast cancer. Taken together, these data support the hypothesis that the invasive phenotype of breast cancer is already genetically programmed at the pre-invasive stages of disease progression.

Box 2

Implications of the DCIS intraductal stress model to BRCA1 and BRCA2

Understanding the origins of genetic instability in the stressful intraductal environment has relevance for chemoprevention and intervention strategies in women who carry a mutated *BRCA1 or BRCA2* gene. The association between BRCA mutations and ductal

carcinoma *in situ* (DCIS) incidence and grade is still limited. Nevertheless, the existing data suggest that DCIS is equally as prevalent in patients who carry BRCA mutations as it is in women who have a high familial risk for breast cancer, but who are not BRCA mutation carriers. However, DCIS, like breast cancer in women with familial BRCA mutations, occurs at an earlier age[102–104] and is often of a high grade (grade III)[102,103]. There is also an increased prevalence of pre-invasive lesions adjacent to invasive cancers in women with familial BRCA mutations[105]. All of these data suggest that BRCA mutation-associated breast cancer progresses through a DCIS precursor at an accelerated pace[102–105] compared with breast cancer arising in patients without a familial BRCA mutation. Mutations in *BRCA1* are associated with genetic instability, increasing the risk of malignant transformation of cells[106]. In an animal model in which *Brca1* is mutated specifically in the mammary epithelium, tumorigenesis occurs in mutant glands after a latency period. Introduction of a *Trp53*-null allele significantly reduces this latency period[106]. Hypoxia increases the stability of hypoxia-inducible factor-α (HIFα) and confers resistance to p53-mediated apoptosis that is induced by genetic damage[12]. Consequently, the hypoxic state of the intraductal DCIS microenvironment will further compound the genetic instability of the DCIS cells in patients with BRCA mutations.

A mouse mammary intraepithelial neoplasia outgrowth (MINO) model of DCIS has also shown that gene expression and genomic changes are present in the initial, pre-malignant MINO lesions[3], and these molecular profiles can be correlated to later phenotypes such as metastasis and responsiveness to oestrogen[7]. This mouse model of DCIS supports the concept that mammary carcinoma aggressiveness is pre-programmed in the pre-cancerous stem cell[3]. Dissociation of the MINO pre-cancerous lesions into three-dimensional spheroid culture revealed a bipotential capacity for myoepithelial and luminal differentiation with the formation of spheroids that could recapitulate the progression to invasive carcinoma following transplantation. By contrast, in comparison to the genetic changes that were found in the MINO DCIS lesions, the additional genetic changes that occur in the invasive carcinomas were subtle, with few consistent changes and no association with phenotype[3,7]. Progression from DCIS to invasive carcinoma was associated with few additional changes in gene expression and genomic organization in the MINO model. The MINO model data support the existence of a pre-cancer stem cell as the origin of invasive breast cancer[3].

Isolation of human malignant DCIS cells

Given the data discussed above, can pre-malignant DCIS spheroid-forming cells be isolated from human DCIS lesions, and do these cells have characteristics that are associated with invasive carcinoma? Our laboratory investigated these questions using *ex vivo* organoid cultures of cells recently isolated from human DCIS lesions[4]. Spheroid-forming cells with an invasive and tumorigenic phenotype were propagated as eight independent patient cell lines. The human DCIS spheroid-forming cells exhibited cytogenetic abnormalities, including gain or loss of portions of chromosomes 1, 5, 6, 8, 13 and 17; invasion of autologous breast stroma *in vitro*; the ability to generate spheroids and duct-like three-dimensional structures in culture; increased expression of proteins associated with autophagy and pro-survival; and the capacity to generate tumours in non-obese diabetic severe combined immunodeficient (NOD/SCID) mice[4].

These cytogenetically abnormal progenitor cells[4] were epithelial cell adhesion molecule (EPCAM)-positive and expressed stem cell markers. It has been postulated that dividing EPCAM-positive mammary progenitor cells can be subject to oncogenic mutations or genetic alterations at different stages during their differentiation[60,63]. The differentiation characteristics of specific invasive breast carcinomas could be a product of the different

subtypes of the epithelial, ductal, alveolar or myoepithelial progenitor cell lineages[8,22,60,63]. We can postulate that such tumour-founding progenitor cells[4,60,64] first arise in a pre-malignant stage such as DCIS. At this early point in the clinical progression of breast cancer the differentiated phenotype and the clinical aggressiveness might be fixed. Therefore, the tumour heterogeneity that is evident under the microscope and in the clinic may be predetermined at the pre-malignant stage[3,5,6,8,22,60,65]. If normal mammary progenitor cells become transformed as they adapt to survive in the high-stress microenvironment of the intraductal niche, then the biology of the resulting cancer is a product of the genetic instability, the suppression of apoptosis and the suppression of DNA repair that arise under stress.

When and why progenitor human cells with pre-existing invasive potential eventually grow out of the DCIS lesion is a central question. The absence of suppressive factors produced by the duct myoepithelial cells, the basement membrane or the stromal cells[66,67] is likely to have a role. Suppressive factors might include myoepithelial cell–glandular epithelial cell interactions; soluble factors secreted by reactive or activated stromal cells, and mesenchymal cells or immune cells; and the barrier function and composition of the extracellular matrix (ECM)[14,17,22,66–69]. However, the activation of autophagy in the neoplastic epithelial cells as a result of stress might also have a central role.

Autophagy: energy source under stress

Autophagy is a pathway that is activated to promote survival in the face of hypoxic and nutrient stress[11,32,33,62,70–74]. Evidence indicates that autophagy is an emerging target for cancer therapy[11,75–79]. Autophagy is upregulated and co-localizes with areas of hypoxic stress in models of epithelial tumours[28]; autophagy-defective, immortalized mammary epithelial cells are more susceptible to cell death under conditions of metabolic stress[26,28]. DCIS growth is limited because of confinement within the duct and the absence of a blood supply. This state can trigger autophagy-mediated survival in the most metabolically stressed regions[11,26,29]. Thus, we can propose that autophagy is a major survival mechanism that is used by DCIS cells to persist and proliferate in the high-stress environment of the intraductal space[4] (FIGS 2,3) and can be a main determinant of DCIS cell fate in response to metabolic stress[11,26,29,44,46,80].

Four metabolic processes link autophagy to the survival and invasion of pre-malignant breast cancer. Hypoxia and nutrient stress are the first link[11,28]. As discussed above, proliferating ductal epithelial cells accumulating within the breast duct do not have access to the vasculature outside the duct. The activation of autophagy could divert the hypoxic DCIS cells away from apoptosis and thereby support the survival and growth of DCIS neoplastic cells within the lumen. Autophagy might also be integral to the removal of dead and dying or necrotic intraductal DCIS cells[44,71,81,82].

Anoikis, the triggering of apoptotic cell death for cells that have been separated from their normal adhesion substratum, is the second link. Normal glandular epithelial cells require attachment to, or association with, the basement membrane ECM for continued survival. During ductal hyperplasia and dysplasia, epithelial cells are a substantial distance away from the peripheral basement membrane. Moreover, invading carcinoma cells can migrate into the stroma in the absence of a basement membrane anchor. Autophagy has been shown to be a key regulator of survival in cells that are deprived of an anchoring substratum, and may have an important role for cell survival in any anchorage-independent state[83].

The third link is matrix degradation. High-grade DCIS, microinvasion and overt invasion are known to be associated with interruptions, remodelling and enzymatic breakdown of the basement membrane and the stromal ECM[5,13,66]. We can also postulate that autophagy

might facilitate cell movement through areas of degraded matrix by processing matrix breakdown fragments that are phagocytosed by the migrating cells[83].

Calcification is the fourth link to autophagy. Microcalcifications are seen in 90% of mammograms[84–86], but microcalcifications of all types are associated with a broad range of breast lesions, and have only a 30–40% specificity for malignancy[13,87]. Nevertheless the shape, size and density of the microcalcification on a mammogram can be a specific indicator of the necrosis that is a characteristic of high-grade DCIS[87–89]. Pleomorphic, small calcifications (<0.5 mm) are also associated with high-grade and intermediate-grade DCIS. By contrast, microcalcifications that are restricted to the lobules are almost always associated with benign breast disease such as microcystic adenosis[13,87,89]. The chemical composition of most DCIS-associated microcalcifications is a subtype of calcium phosphate, hydroxyapatite, which is easily detectable by conventional light microscopy. The individual calcifications often appear concentrically layered, giving the impression that calcium deposition is accumulating over time. Calcium phosphate deposition is associated with the accumulation of necrotic cellular material[13], and has been documented during the necrosis of fat cells in the breast[13]. As such, microcalcifications could provide an important clue about the developmental age of intermediate-grade and high-grade DCIS lesions. As hypoxia-induced necrosis might well precede calcium deposition, and calcium deposition occurs over time, we suggest that most intermediate-grade and high-grade DCIS lesions are subjected to hypoxic stress for a long period of time before diagnosis[13,24]. Thus, microcalcifications might be a signature of ongoing hypoxic stress and conditions that favour genetic instability. Although microcalcifications are a consequence and not a primary cause of DCIS they might contribute to the persistence of the DCIS lesions. Insoluble calcium has been shown to induce autophagy[90], and may contribute to the local oxidative or metabolic stress within the duct.

Anti-autophagy: a new clinical strategy

The ability to isolate and grow human DCIS progenitor cells as spheroids in culture[4] has made it possible to test whether autophagy has a role in DCIS progenitor cell survival[4]. DCIS spheroid-forming epithelial cells have increased expression of proteins associated with autophagy[4,26,32,46] (FIG. 3) that persist in culture and in tumours generated by these cells in NOD/SCID mice[4]. Chloroquine, a clinically well-studied compound, is an orally administered small-molecule inhibitor that arrests the autophagy pathway by disrupting the cellular lysosomal function[32,73] and altering the fusion of autophagosomes with lysosomes. Treatment of DCIS progenitor spheroids in culture with chloroquine reduced the expression of the autophagy-associated proteins, killed the DCIS progenitor spheroids and prevented tumorigenicity in NOD/SCID mice[4]. On the basis of these findings we suggest that autophagy promotes the survival of cytogenetically abnormal human DCIS malignant progenitor cells[4]. Consequently, the disruption of lysosomal function, or the abrogation of autophagy, constitute novel targets for treating DCIS. Understanding the regulation of autophagy provides a fertile field of therapeutic strategies for chemoprevention[33,45,46].

The safety profile of oral chloroquine has been established through its use worldwide as an acute therapy for malaria[91–95]. Chloroquine can suppress *N*-methyl-*N*-nitrosurea-induced mouse breast carcinongenesis, increase the effectiveness of tyrosine kinase inhibitor treatment of primary chronic myeloid leukaemia stem cells, and has been proposed as a therapy for MYC-induced lymphomagenesis because it induces lysosomal stress and causes p53-dependent and caspase-independent cell death[75,76,92,96]. Treatment with chloroquine has also been proposed as a potential means to increase the effectiveness of tamoxifen in oestrogen receptor-positive (ER^+) breast cancer by enhancing cell death in the sub-population of tamoxifen-resistant breast cancer cells that emerges during treatment[79]. ER^-

breast cancer cells, in which tamoxifen therapy is contraindicated, may also exhibit chloroquine-induced cell death by blocking autophagy-dependent cell survival[75,76,92]. Thus, there is a rationale for the use of chloroquine in a patient with DCIS whose lesion is ER^+ or ER^-.

Chemoprevention strategies for the future

Tamoxifen is currently the only approved systemic treatment for preventing the recurrence of ER^+ DCIS[21,97,98]. Tamoxifen was assessed in an investigational neoadjuvant study of ER^+ DCIS where it was administered after diagnosis using a primary biopsy but before the commencement of standard-of-care surgical therapy[99]. This tamoxifen DCIS neoadjuvant study has established a precedent trial design for neoadjuvant therapy of DCIS.

We have recently opened a clinical trial of neoadjuvant therapy in patients with DCIS that provides a model system for testing any new and toxicity-tested compound for its ability to kill human pre-malignant DCIS progenitor cells. The trial will directly test the hypothesis that the disruption of autophagy-dependent lysosomal function by chloroquine is an effective treatment for DCIS. Chloroquine in this study is used as a short-term treatment after primary diagnosis but before surgical therapy. Previous animal studies have indicated that chloroquine treatment can enhance therapy-induced tumour apoptosis[73,75,76], which can create increased numbers of necrotic cancer cells[74], generating an inflammatory response. Theoretically, the resulting necrosis-induced inflammation over long-term chloroquine therapy may promote tumour progression[74]. We propose that a short exposure to chloroquine, before surgery, will prevent this potential side effect.

Patients with ER^+ high-grade DCIS will receive standard-of-care tamoxifen plus chloroquine. Patients with ER^- high-grade DCIS (expected to be approximately 50% of the high-grade DCIS cases) will receive chloroquine alone. Each patient is subject to magnetic resonance imaging (MRI) before enrolment and just before surgery (mastectomy or lumpectomy depending on the size and confluence of the primary DCIS lesion) 3 months after treatment (FIG. 4). Efficacy of the short-term 3-month therapy with either tamoxifen and chloroquine or chloroquine alone in this distinctive DCIS trial design will be uniquely measured directly at the molecular level. The genotype and the phenotype of harvested DCIS malignant progenitor cells, and the molecular histology of the DCIS lesion will be compared with MRI images before and after the 3-month course of therapy. This type of trial design could be used to test new agents for the ability to arrest breast cancer invasion at the pre-malignant stage[100,101]. We imagine a future in which a limited course of low toxicity therapy is administered to suppress or eradicate pre-malignant breast lesions in high-risk patients, even if the pre-malignant lesions are undetectable by standard imaging.

Conclusion

New strategies for killing DCIS cells, which remove their ability to survive in the stressful intraductal space, have led to an ongoing neoadjuvant therapy trial for DCIS. Treating breast cancer before it can become invasive is analogous to the prevention of cervical cancer by treating pre-malignant cervical dysplasia, or to the prevention of colon cancer by the removal of pre-malignant polyps[100,101]. The knowledge gained from this new class of DCIS treatment trials might provide a basis for therapies aimed at suppressing or eradicating pre-malignant breast lesions.

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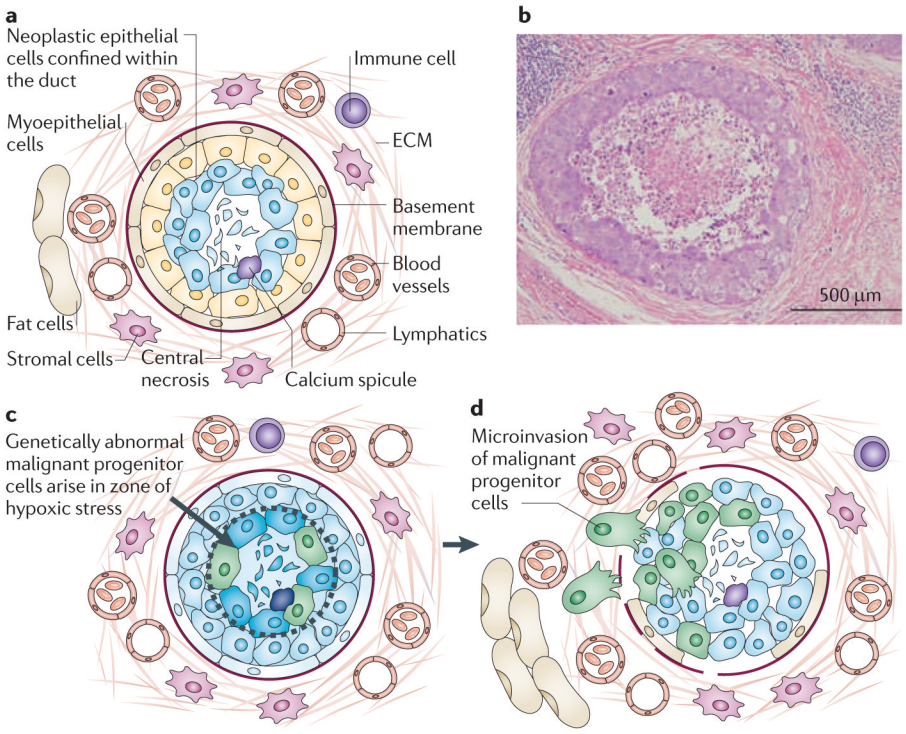

Figure 1. The stressful microenvironment of the intraductal niche may promote genetic instability

a | This figure shows the microecology of ductal carcinoma *in situ* (DCIS) lesions in the breast. DCIS neoplastic cells proliferate within the duct, which is bound by the basement membrane and a rim of myoepithelial cells on the lumen side of the basement membrane. The normal duct is composed of a single epithelial layer. By contrast, the DCIS lesion contains multiple layers of cells that accumulate inwards into the lumen. Outside the basement membrane the breast stroma contains the extracellular matrix (ECM), lymphatics, blood vessels, stromal cells, immune cells and fat cells. All of these components participate in the carcinogenic process by promoting or suppressing malignant progenitor cells that could arise within the mass of cells accumulating in the duct. **b** | Haematoxylin and Eosin stained section (original magnification x10) of a comedo-DCIS lesion, exhibiting central necrosis, stroma and lymphocytes. **c** | Necrosis occurs in the centre of the duct because these cells are the furthest radial distance from the oxygen and nutrients that diffuse from the vessels outside the duct. Proliferating cells within the intraductal microenvironment are under high stress because they are nutrient deprived, hypoxic, crowded and undergoing oxidative stress. **d** | Mammary pluripotent stem cells must adapt to survive in the high-stress microenvironment. Adaptation promotes the suppression of apoptosis in the face of genetic instability and could lead to the generation of genetically abnormal malignant progenitor cells before the onset of invasion. Invasion is associated with the loss of myoepithelial cells, periductal angiogenesis, fragmentation of the basement membrane and chemotaxis radially outwards.

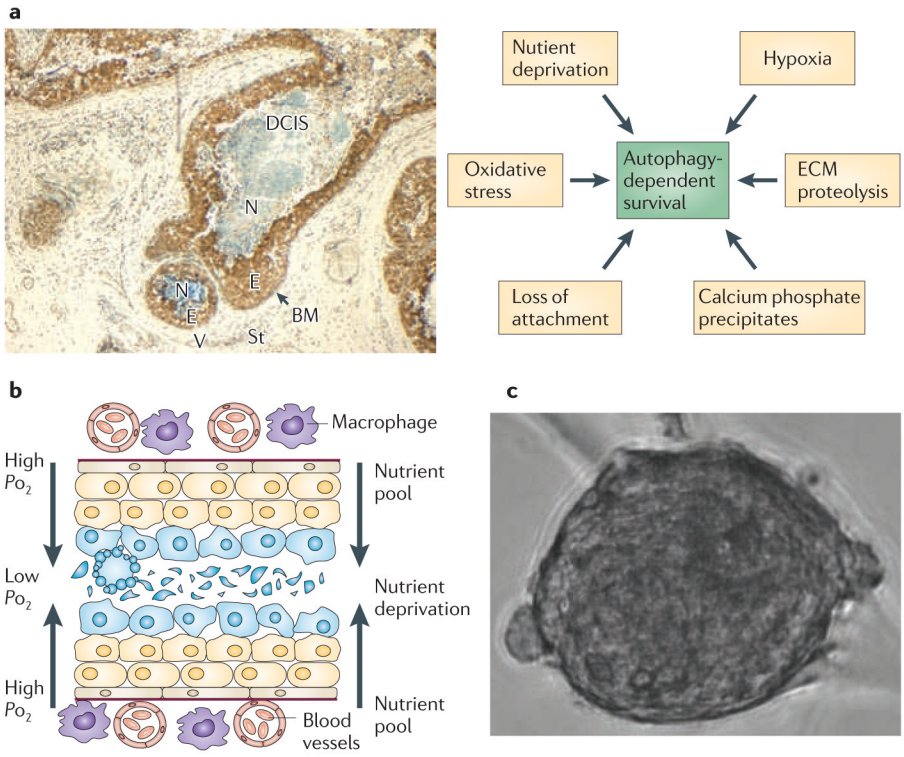

Figure 2. Autophagy and cell survival in DCIS lesions

a | Human comedo-ductal carcinoma *in situ* (DCIS) lesion (original magnification x10) stained with antibodies to Beclin 1 shows upregulation of Beclin 1 (brown staining) in the viable rim of intraductal cells[4] within the hypoxic ductal niche. Multiple types of stress that impinge on the DCIS cells directly contribute to the activation of autophagy (arrows; right side of figure). **b** | As abnormal DCIS cells accumulate within the duct many are pushed further from the source of oxygen and nutrients outside the duct. This sets up a gradient of stress that could be a 'breeding ground' for the mutational and cytogenetic abnormalities that drive breast cancer. Understanding how genetically abnormal DCIS cells arise and survive within the high-stress microenvironment provides targets for chemoprevention. **c** | Example of a human malignant precursor spheroid that can be propagated from fresh DCIS tissue[4]. BM, basement membrane; E, epithelial cells; ECM, extracellular matrix; N, necrosis; St, stroma; V, vessels.

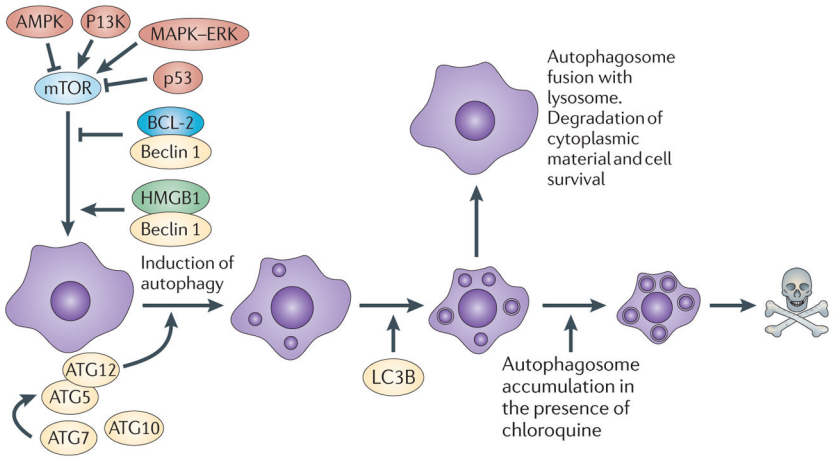

Figure 3. Upstream pathways that intersect with the autophagic pathway

Autophagy is activated and regulated by various molecules that are known to be involved in oncogenesis, such as AMP-activated protein kinase (AMPK), PI3K and p53. Autophagy is a catabolic process involving the degradation of the components of a cell by its own lysosomal machinery. Autophagy is a major mechanism by which a starving cell reallocates nutrients from unnecessary processes to more essential processes. The best-studied mechanism of autophagy involves the nucleation of a double-membrane vesicle around a cellular constituent. The resultant vesicle then fuses with a lysosome that contains acid proteases that degrade the contents, ultimately generating ATP for the cell. Chloroquine treatment blocks autophagy by interfering with the fusion of the autophagosome vesicle with the lysosome and by altering the acidic internal pH of the lysosome. ATG12, autophagy 12; HMGB1, high mobility group protein B1; LC3B, microtubule-associated protein 1 light chain 3β.

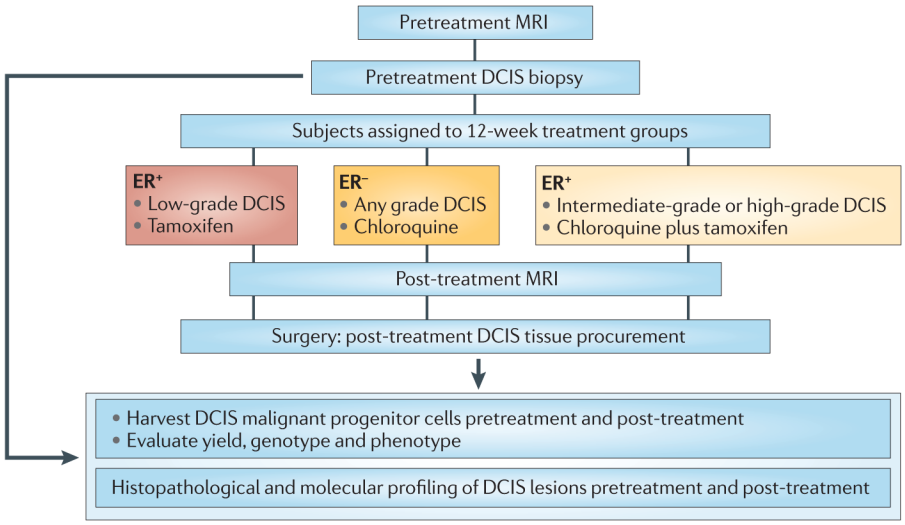

Figure 4. A neoadjuvant therapy trial for DCIS

The Preventing Invasive Neoplasia with Chloroquine (PINC) trial (NCT01023477; see Further information) examines the safety and effectiveness of chloroquine administration to patients with low-grade, intermediate-grade or high-grade ductal carcinoma *in situ* (DCIS). Patients with high-grade DCIS whose lesion is oestrogen receptor-positive (ER^+) will receive standard of care tamoxifen plus chloroquine. Patients who are ER^- will receive chloroquine alone. Patients with ER^+, low-grade lesions will receive tamoxifen only. Magnetic resonance imaging (MRI) will be carried out on each patient before enrolment and just before the standard-of-care surgical therapy, which will be mastectomy or lumpectomy depending on the size and confluence of the primary DCIS lesion. Effectiveness in this DCIS trial design will be uniquely measured directly at the molecular level in the DCIS tissue before and after treatment. The genotype, phenotype and proteome of the harvested malignant progenitor cells from the DCIS lesions, and the molecular histology of the DCIS lesion, will be compared with MRI images before and after a 3-month course of therapy.