Three-dimensional extracellular matrix culture models of EGFR signalling and drug response

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Abstract

Three-dimensional extracellular matrix culture, on substrata such as Matrigel, restores many aspects of the differentiated state to non-malignant cells from a variety of tissues. We have adapted these techniques to study EGFR (epidermal growth factor receptor) signalling and drug response in breast cancer cell lines. EGFR-dependent breast cancer cell lines undergo a striking reversion of the malignant phenotype upon treatment with inhibitors targeting the receptor, or downstream signalling intermediates such as mitogen-activated protein kinase and PI3K (phosphoinositide 3-kinase). Using this approach, we have recently reported that EGFR signalling in breast cancer can be effectively inhibited by blocking the activity of a key protease, TACE [TNFα (tumour necrosis factor α)-converting enzyme], which regulates the bioavailability of EGFR ligands. These results suggest a new way to target EGFR signalling in tumours of the breast and other epithelial tissues and underline the value of three-dimensional extracellular matrix culture models for exploring cancer-relevant signalling processes *ex vivo*.

Introduction

Studying cells *ex vivo* is a cornerstone in our approaches to understanding physiological and pathological processes. However, one of the fundamental problems with this approach is that, upon dissociation and culture on tissue culture substrata, cells rapidly lose many of the hallmarks of their differentiated function *in vivo* [1–4]. Accordingly, the relevance of what is being studied in culture must always be questioned. One of the prominent differences between tissue culture plastic and the *in vivo* epithelial microenvironment is the presence of basement membrane, a thin layer of extracellular matrix proteins comprising laminins, collagen IV, nidogen, entactin and proteoglycans [5].

Over the past three decades, much evidence has accumulated to indicate that components of the extracellular matrix provide key structural and biochemical cues that are key to the maintenance of the differentiated state in non-malignant mammary epithelial cells [6–11]. Following the premise that studying cancer in this way is likely to yield more valuable information than growing cells on two-dimensional substrata, we have adapted these methods to study a large panel of human breast cancer cell lines [12,13]. As we have shown for the mouse, important differences in the expression patterns of genes and proteins are observed – even in malignant human breast cancer cell lines – upon culture in these more physiologically relevant substrata.

Key words: breast cancer cell line, drug response, epidermal growth factor receptor (EGFR), three-dimensional extracellular matrix culture, tumour necrosis factor α (TNFα), tumour necrosis factor α-converting enzyme (TACE).

Abbreviations used: ADAM, a disintegrin and metalloproteinase; EGF, epidermal growth factor; EGFR, EGF receptor; lrECM, laminin-rich extracellular matrix; PI3K, phosphoinositide 3-kinase; siRNA, small interfering RNA; TGFα, transforming growth factor α; TNFα, tumour necrosis factor α; TACE, TNFα-converting enzyme.

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Three-dimensional culture models of breast cancer cells

We have extensively studied a culture model of human breast cancer progression (HMT-3522) consisting of non-malignant (termed S1, [14]) and malignant (termed T4-2, [15]) breast epithelial cells derived from the same individual. The S1 cells require EGF (epidermal growth factor) for proliferation, and among the hallmarks of the malignant phenotype acquired by T4-2 cells is the ability to proliferate in the absence of EGFR (EGF receptor) ligands. Nevertheless, the T4-2 cells remain dependent on the activity of the EGFR pathway as their growth may be inhibited by using either small molecule inhibitors of EGFR or antibodies that block this receptor [16]. This exquisite dependence on flux through the EGFR pathway makes HMT-3522 an ideal model system to study the regulation of this pathway in cancer cells.

When S1 (and other non-malignant breast epithelial cells) are cultured in three-dimensional extracellular matrix cultures they undergo a few rounds of cell division before forming a polarized, growth-arrested colony that is reminiscent of a breast acinus *in vivo* [13]. In contrast, T4-2 (and other malignant breast epithelial cells) form disorganized, continuously proliferating colonies [12,13]. This three-dimensional extracellular matrix culture model serves to distinguish rapidly between malignant and non-malignant cells. In addition, striking morphological responses can be observed when key signalling pathways upon which particular tumour cells depend are inhibited. For example, in T4-2 cells, inhibition of β1-integrin [17] or kinases in the EGFR pathway [18,19] results in a pronounced reversion of the malignant phenotype in cells grown in three-dimensional culture: these cells form small, round growth-arrested multicellular colonies in which many hallmarks

Figure 1 | Reversion of the malignant phenotype of breast cancer cell lines in three-dimensional Matrigel culture

Untreated T4-2 cells form continuously proliferating disorganized colonies (left). Treatment with inhibitors of EGFR (80 nM AG1478 or 4 μg/ml mAb225) restores the normal mammary acinar morphology to these malignant cells.

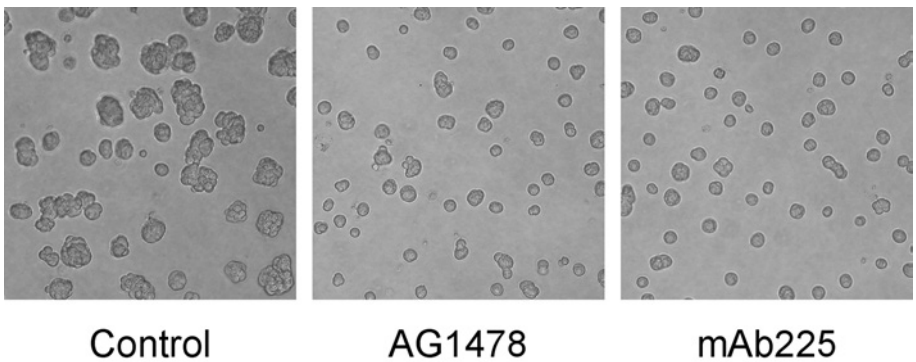
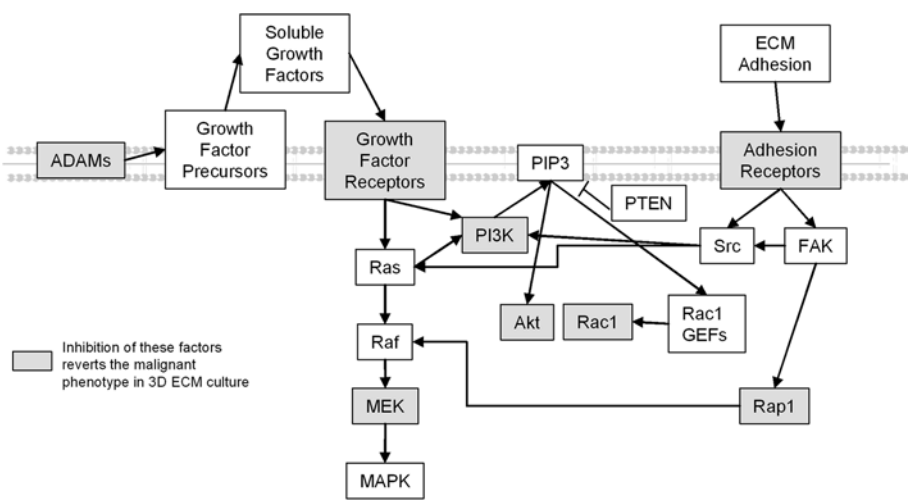


Figure 2 | Schematic representation of the signalling pathways downstream of growth factor and adhesion receptors in T4-2 cells

The activity of some key proteins (grey boxes) is required for the maintenance of the malignant phenotype. Inhibition of these key signalling nodes is sufficient to revert the malignant phenotype in the manner shown in Figure 1. Reproduced with permission from [38]. © 2005 Cold Spring Harbor Laboratory Press.

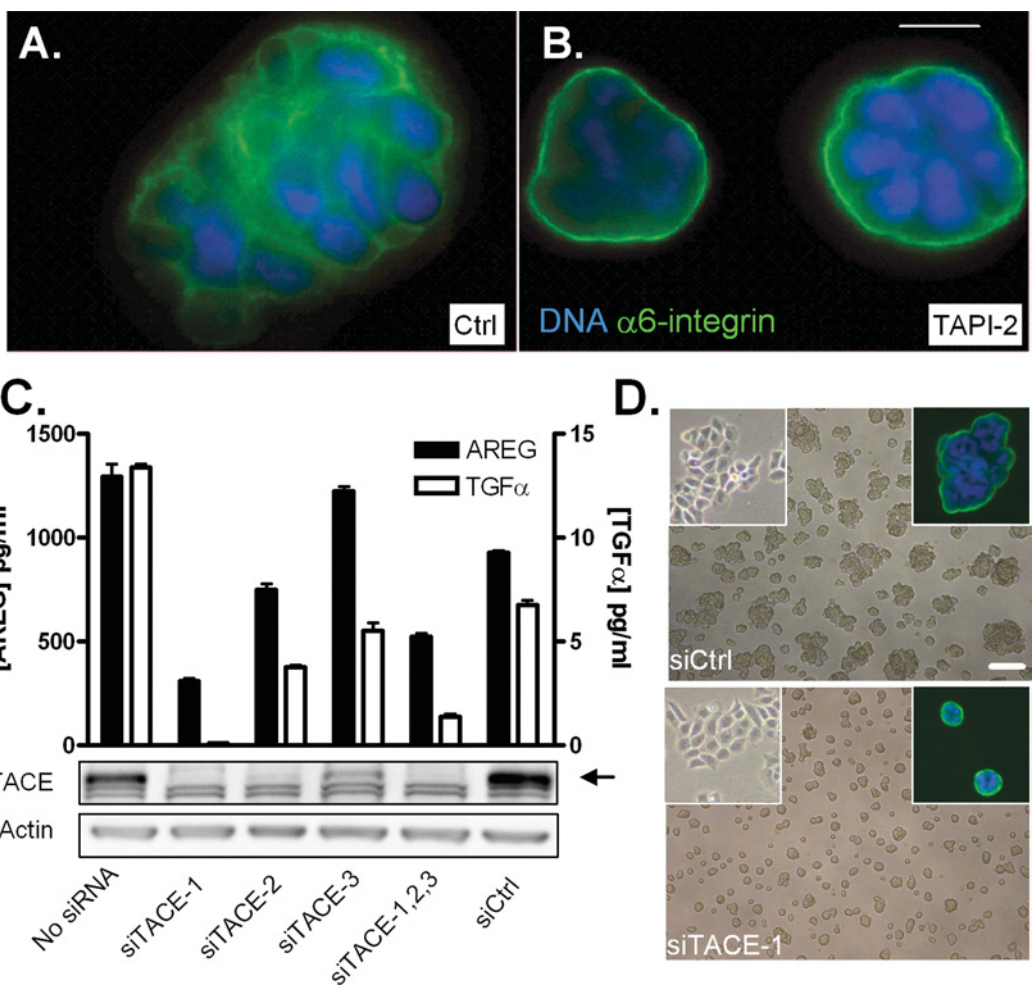


of mammary acinar organization are restored (for reviews see [20,21]). A representative example is shown of T4-2 cells treated with two agents targeting the EGFR: a small molecule inhibitor, AG1478, and a blocking antibody, mAb225 (Figure 1).

Collectively, these studies have shown that signal transduction pathways in non-malignant cells are integrated in three-dimensional lrECM (laminin-rich extracellular matrix) cultures in ways not observed when cells are cultured as monolayers. Upon inhibition of either EGFR or β1-integrin, the expression and activity of the other protein is down-regulated in T4-2 cells cultured in Matrigel but not, crucially, on two-dimensional plastic substrata [16]. T4-2 cells are also sensitive to inhibition of PI3K (phosphoinositide 3-kinase), which results in the reversion of the malignant phenotype with concomitant down-regulation of EGFR, β1-integrin and up-regulation of PTEN (phosphatase and tensin homologue deleted on chromosome 10) in cells cultured on Matrigel. Factors directly downstream of PI3K, such as Akt and GSK3β (glycogen synthase kinase 3β), were inhibited by this treatment on both culture substrata, indicating that the drug is effective under both conditions but the propagation of the effect through the signalling network is different. These results have raised the question of the extent to which monolayer cultures may be failing to recapitulate signalling *in vivo* [20,22]. Recently, the small GTPase Rap1 has also been shown to be involved in promoting the malignant phenotype in T4-2 cells, and its inhibition resulted in the restoration of the normal mammary acinar architecture [23]. Using these approaches, we have begun to map the key signalling nodes at which the use of specific inhibitors can interrupt tumour cell proliferation in this breast cancer model (Figure 2).

Figure 3 | Inhibition of TACE-dependent shedding of EGFR ligands reverts the malignant phenotype of T4-2 cells

(**A**) T4-2 cells grown in three-dimensional lrECM cultures form continuously proliferating, disorganized and apolar colonies. α6-integrin staining of vehicle-treated T4-2 cells indicates absence of tissue polarity. (**B**) T4-2 cells treated with a broad-spectrum MMP (matrix metalloproteinase)/ADAM inhibitor (20 μM TAPI-2) undergo a morphological reversion similar to that of EGFR inhibitor-treated cells. α6-Integrin staining of TAPI-2-treated T4-2 cells shows restoration of tissue polarity. Scale bar, 100 μm. (**C**) ELISA analysis of EGFR ligand shedding in T4-2 cells transfected with three siRNA oligonucleotides, either individually or as a pool. Ligand shedding is proportional to the level of TACE expression. (**D**) Reversion of the malignant phenotype of T4-2 cells in three-dimensional lrECM culture following transfection of siRNA against TACE. Left insets: phase-contrast micrographs of transfected cells grown on plastic. Right insets: α6-integrin immunostaining of representative colonies. Modified with permission from [24].



Regulation of the EGFR pathway in HMT-3522

We have recently described the mechanism by which T4-2 cells have acquired the ability to proliferate in the absence of exogenous growth factors and, in so doing, developed an interesting new way to target the EGFR pathway in breast cancer. As T4-2 cells lack mutations in commonly mutated proto-oncogenes (such as Ras, Raf, EGFR and *PIK3CA*) we screened for production of EGFR ligands and determined that T4-2 cells overexpress two of these proteins, Amphiregulin and TGFα (transforming growth factor α) [24]. Like all of the other ErbB ligands, these proteins are initially synthesized as transmembrane precursors which need to be liberated from the cell surface so that they can interact with receptors on the producing cell or on nearby cells [25]. The protease which sheds both Amphiregulin and TGFα is TACE [TNFα (tumour necrosis factor α)-converting enzyme], also known as ADAM17 (a disintegrin and metalloproteinase 17) [26,27]. We and others have shown that targeting TACE pharmacologically is a viable way of inhibiting the EGFR signalling pathway in squamous cell carcinoma [28,29], non-small cell lung cancer [30], renal carcinoma [31] and breast cancer [24]. In most of these studies, the emphasis has been placed on the suppression of growth

factor mobilization, although it is clear that other factors, such as the suppression of growth factor receptor cleavage (which can result in a constitutively signalling receptor), may also make a contribution, at least in certain systems [30].

In T4-2 cells, treatment with a small molecule inhibitor of TACE, TAPI-2, resulted in a reversion of the malignant phenotype and the restoration of the normal mammary acinar architecture (Figures 3A and 3B). Specific suppression of TACE using siRNAs (small interfering RNAs) resulted in a prevention of the shedding of both EGFR ligands (Figure 3C) and recapitulated the effects of the more broad-spectrum small molecule inhibitor in the three-dimensional culture assay (Figure 3D). We have also reported that TACE and TGFα are expressed at high levels in human breast cancer patients with a poor prognosis, and that they are particularly highly expressed in breast cancers of the 'basal' subtype [24]. Because of the role of TACE as the primary sheddase of TNFα [32,33], several pharmaceutical companies have developed inhibitors of this enzyme [34–36] as potential therapy for rheumatoid arthritis, a disease in which TNFα plays a prominent role. These compounds could also be tested for their ability to inhibit the EGFR pathway in cancer patients. One pharmaceutical company, Incyte Corporation, has reported testing TACE inhibitors in *in vivo* cancer models with very encouraging results [30,37], although whether this approach will prove successful in human patients remains to be determined.

Conclusions

Studying cells in the context of physiologically relevant extracellular matrices offers the potential to explore signalling processes in ways that more closely approximate the *in vivo* microenvironment. These approaches have enormous value for the identification and dissection of the key molecular vulnerabilities of cancer cells and the testing of targeted therapies against these factors. These systems are gaining widespread appreciation for their utility in the study of tumour cell autonomous processes and are likely to prove invaluable for the investigation of the heterotypic interactions between tumour cells and other cell types of the tumour microenvironment in more complex co-culture models.

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Received 19 March 2007