Breast Cancer Metastasis Studying Tool

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During the metastatic process, breast cancer cells must come into contact with the extra-cellular matrix (ECM) at every step. The ECM provides both structural support and biochemical cues, and cell–ECM interactions can lead to changes in drug response. Here, we used fibroblast-derived ECM (FDM) to perform high throughput drug screening of 4T1 breast cancer cells on metastatic organ ECM (lung), and we see that drug response differs from treatment on plastic. The FDMs that we can produce from different organs are abundant in and contains a complex mixture of ECM proteins. We also show differences in ECM composition between the primary site and secondary organ sites. Furthermore, we show that global kinase signalling of 4T1 cells on the ECM is relatively unchanged between organs, while changes in signalling compared to plastic are significant. Our study highlights the importance of context when testing drug response in vitro, showing that consideration of the ECM is critically important.

fibroblast-derived matrix cancer drug screening

1. Introduction

Metastatic disease contributes to 66–90% of cancer deaths [1][2]; therefore, understanding the complex interactions of cancer cells and their microenvironment at the primary and metastatic sites will be vital in providing effective treatment. The process of metastasis is multi-step, complex, and significantly driven by the microenvironment. Cells are embedded in a 3D scaffold within tissues known as the extra-cellular matrix (ECM) [3]. Cell-ECM interactions play a fundamental role in the proper regulation of cell behaviour and homeostasis, especially in response to environmental changes and stresses [4]. In particular, cancer-cell–ECM interactions are critical for metastasis since cancer cells must interact with ECM at every stage of the metastatic cascade, eventually attaching to the ECM of the secondary organ to initiate growth; the so-called seed (cancer cells) and soil (organ ECM) hypothesis [5].

The tumour microenvironment is comprised of many different cell types, which together interact and regulate the ECM. Fibroblasts are highly involved in the production and remodelling of the ECM in vivo, and during normal development and physiology, these cells produce mainly connective tissue ECM [6][7]. Specifically, ECM at distinct organ niches is different in composition, structure, and biophysical properties, often related to function in normal physiology. The ECM composition of the lung, for example, is rich in fibrillar collagens and elastin, which are important to allow the necessary ‘recoil’ [8]. At the primary site in breast cancer, the tumour’s ECM is often stiffer [9] and characterised by an increase of collagen I remodelling and deposition, which facilitates local invasion due to increased linearisation of fibres [10]. During tumorigenesis, resident fibroblasts become ‘activated’, similar to when

they are activated during normal wound healing [11]. The global composition of the ECM has been catalogued using both in silico and biophysical approaches leading to what is known as the ‘matrisome’; a database containing 1056 genes describing 278 ‘core’ ECM proteins [12]. Recently it has become possible to produce native ECM from fibroblasts in vitro and use the ECM that they produce in cancer investigations [13].

Cells interact with the ECM via integrins and glycoproteins, which in turn transduce signals internally, leading to the activation of intracellular kinases [4]. Kinase activation via protein phosphorylation within cells is a key post-translational modification that is involved in the regulation of intracellular signalling, which leads to the induction of gene expression, metabolic changes, proliferation, differentiation, and membrane transport [14]. Over 500 kinases are identified in the human genome [15] and are divided into two categories: tyrosine kinases (PTKs) and serine/threonine kinases (STKs) [16]. Tyrosine kinases transfer a phosphate molecule from ATP to the tyrosine residue [17]. Aberrant activation of receptor tyrosine kinases has been described in carcinogenesis, examples of which include the overexpression of human epidermal growth factor receptor (EFGR) and platelet-derived growth factor receptor α/β (PDGFR α/β) [18][19]. There are currently (as of September 2020) 52 receptor tyrosine kinase inhibitors currently approved by the US-FDA, 46 of which are used in the treatment of neoplastic diseases [20], indicating an increased use and focus within cancer treatment.

There has been an increasing number of in vitro approaches to anti-cancer drug testing, often balancing increasing complexity that better represents tumours and the ability to screen at a high throughput level. These approaches range from simple viability assays, where cancer cells on plastic are treated with a prospective compound, and cytotoxicity is measured [21], to complex patient-derived 3D multicellular cultures [22]. Issues with cancer cells treated on a two-dimensional monolayer are that they often do not reflect complex three-dimensional tissue architecture or cell–ECM interactions [23]. However, with increasing complexity and dimensionality, issues with maintenance and control during testing lead to decreased screening capacity. Therefore, it is crucial to continue to investigate new in vitro experimental approaches that consider the importance of the tumour microenvironment and especially the ECM in anti-cancer drug testing.

Many breast cancer types have a poorer clinical outcome if the disease has progressed to stage IV (metastatic disease) [24][25]. Breast cancer typically metastasises to the lymph nodes, lungs, liver, bones, and brain, and currently, treatment options for distant metastasis include surgery, radiation, and a small number of chemotherapeutic agents [26]. However, these treatments often have limited efficacy and impact on patient survival.

In this study, we have focused on breast cancer and used the highly metastatic 4T1 mouse mammary carcinoma cell line (and 67NR non-metastatic counterpart) [27], which readily metastasises to lymph nodes, lungs, and liver, and most closely resembles the basal/TNBC (triple-negative breast cancer) breast cancer subtype [28]. Here, we have used fibroblasts isolated from mouse lymph node, lung, and mammary gland tissue to generate 3D fibroblast-derived matrices (FDMs) that closely resemble in vivo mesenchymal matrices [13][29]. We can use these FDMs to expose breast cancer cells to different ECM organ environments (primary and metastatic sites), analyse induced global kinase signalling in the cancer cells, the proliferation of cancer cells, and importantly, conduct a large-scale drug screening.

In this study, we show that despite differences in FDM composition between primary and metastatic sites, global kinase signalling induced in breast cancer cells on FDMs remains relatively unchanged between different organ ECMs. We observe that when exposed to the ECM, 4T1 breast cancer cells induce different global kinase signalling signatures comparative to plastic, which correlates with drug response. Importantly, we reveal that the drug response differs significantly between cancer cells treated on the FDMs and plastic, calling into question the relevance of using plastic for large-scale drug screening in the pre-clinical setting. We show that while the changes in lung/mammary FDM we produce overlap nicely with in vivo protein composition of the lung vs. mammary decellularised organs, we can elucidate similar responses in kinase signalling and drug response when breast cancer cells are exposed to the 'simpler' commercially available matrices. This paves the way for simple improvements in drug testing that considers the important role of the ECM.

2. Current Analysis on Cancer–ECM interactions

Knowing the important role that the ECM plays during metastatic progression of breast cancer, we adapted a cell-based approach to derive in vitro native ECM and investigated kinase signalling and drug response in a model of metastatic breast cancer. We used normal fibroblasts from known metastatic organs in breast cancer (lymph nodes and lungs) as well as the primary site (mammary gland). These FDMs closely resemble in vivo the ECM components of decellularised lungs vs. mammary, and we show by mass spectrometry that the FDMs generated are complex and abundant in ECM constituents.

Cancer–ECM interactions are critical during metastatic progression. In order for cancer cells at the primary tumour site to travel to local and distant organs, they must come in contact with and move through the ECM within and surrounding the primary tumour (invasion), followed by breaching of the basement membrane (intravasation), and then the reverse at the secondary site (extravasation) [30]. At all times, the ECM is providing biophysical support and biochemical cues that affect the ability of cancer cells to proliferate and invade, which could be targeted by therapeutics [31]. The ECM during cancer progression is often altered in terms of stiffness, structure, arrangement, and composition [4] and as such, provides a unique microenvironment in which cancer cells can activate intracellular signalling pathways necessary for proliferation and migration. We also see that our mammary FDMs have significantly increased collagen I levels compared to the lung FDM, which recapitulates the in vivo situation, as our previous proteomics also shows an increase in collagen I. Increasingly, 'simpler' ECM components have also been the focus of cancer studies to include a relevant tumour microenvironment. However, they are often limited in their ability to recapitulate these cancer niche properties as products such as Matrigel and gelatin are only composed of a limited number of ECM components and lack resemblance to native tissue architecture and biophysical properties [32]. The presence of the ECM is also critical when studying cancer cell behaviour in vitro to aid in increasing the efficacy of drugs tested in vitro to patients, as current studies suggest fewer than 5% of all drugs tested make it to the clinic [33][34]. To our knowledge, using FDMs to investigate global kinase signalling and for use as a high throughput drug screening platform has not been previously performed.

We show that irrespective of which organ-specific FDM the 4T1 breast cancer cells are in contact with, overall, there are only minor changes in kinase activation and de-activation. Cancer cells bind to the ECM through integrins

and glycoproteins, which transduce signals from the tumour microenvironment into the cell. Integrins are versatile transmembrane proteins that are able to bind to different ECM components such as collagen, laminins, and fibronectin [35]. We therefore posit that if there are a range of different ECM components present surrounding the cancer cells, integrin-mediated kinase signalling can be induced. Further in-depth investigations looking at which specific ECM components (or redundancy in the system) induce key signalling pathways, and through which receptors signalling occurs, will be very beneficial to better understand the ECM-cancer cell interaction in breast cancer. While not directly studied here, the role of specific, important signalling pathways, such as those related to TGFβ, are also likely to be altered and contribute to the results we see. TGFβ has been shown to activate fibroblasts within the tumour microenvironment and therefore induce the production of lysyl oxidase, via SMAD, leading to increased collagen crosslinking and metastasis in breast cancer [36]. We see that both in the mammary and lung FDM, lox is significantly increased in lung vs. mammary. Furthermore, it would be beneficial in this system to further explore whether other functions of the cancers cells are altered via looking at migration and invasion. Multiple studies have shown [37] that FDMs can be used to perform migration studies as they produce the necessary collagen structures to allow this [13][38].

The organ-specific fibroblasts that we used in this study were derived from resident fibroblasts that are present within the organ of healthy mice. While this gives us a good opportunity to look at differences in inter-organ composition during tumour progression (when cancer cells arrive at a new organ) and kinase signalling, during breast cancer progression, cancer cells are in contact with CAFs as well as resident fibroblasts. CAFs have been shown to remodel and reorganise the ECM, provide tracks for cell migration, and physically pull cancer cells through the tumour microenvironment, aiding in cancer dissemination [7]. We see that when on the mCAF1 FDM, kinase signalling is different to the other FDMs and observe that numerous kinases were de-activated on mCAF1 FDM compared to plastic. Furthermore, we saw that there were no mCAF1 FDM-specific inhibitors. Our study therefore highlights the need for further investigations including the difference between CAF and normally secreted FDM in order to provide better in vitro drug testing and kinase signalling platforms. We also hypothesise that the ECM deposited by the mCAF1s leads to lowered kinase activation levels across many kinases that are important for cell proliferation (such as cyclin-dependent kinases). When these levels are ‘lowered’, drugs that target kinases which are normally highly activated in cancer cells are no longer as efficacious. It would therefore be of interest to see whether the use of combination treatments of kinases inhibitors and PP2A (Protein Phosphatase 2) activators such as SMAP (small molecule activator of PP2A), that allows the re-activation of kinase signalling pathways [39], could lead to re-sensitisation to certain kinase inhibitors and increased efficacy.

We have shown that 4T1 cells on different ECM types display only subtle changes in cell response, despite differences in ECM composition. However, we observe very large differences in both kinase signalling and, more importantly, drug response when 4T1 cells are seeded on plastic. We show that distinctive kinase signalling pathways are activated in 4T1 breast cancer cells and 67NRs depending on whether they are on ECM or plastic, while comparatively minor signalling changes were observed in response to FDMs of different composition. Kinases that could be used as targets at the metastatic lung site would might be EphA5, Tyro3, Ron, TRKB, CK2alpha, and Pim3; however, these kinases display lower changes in activation in the lung compared to mammary. Kinases that would be of interest, as targeting possibilities for metastasis in general in breast cancer

would be those that are activated on the FDMs vs. plastic, and in the 4T1, such as ITK, IRR, PKCα/δ/γ/φ/ε, PKG1/2, Pim1, PKD1, RSK1, IKKα/β, ANP, CK1, MAPKAPK2/3, Akt1/2, CamK4, p7056kβ, SGK, PDGFRα/β, FGFR, pKA, and PRKX. These could also be further investigated as biomarkers in breast cancer, as changed expression levels may correlate with metastasis-free survival, particularly to the lungs.

Conventional drug screening platforms performed in vitro are often 2D and use plastic as a substrate. Increasingly, pre-clinical cancer research is moving towards using models which are 3D and encompass the greater complexities of the tumour microenvironment to better model diseases and test drugs. However, progress in this area is hindered as platforms such as these are difficult to 'scale-up'. Matrigel-based approaches to drug screening are common in organoid cultures, where primary cells from patient tumours are embedded within 3D Matrigel. While these cultures often maintain the genomic properties of the tumours, and encompass cell heterogeneity, they have can be difficult to maintain [40]. While other Matrigel-based cultures such as MBOC (Matrigel bilayer organoid culture) developed for gynaecological tumours [41] have been shown to improve the viability of cell cultures and allow for drug screening, they yet do not encompass the complex mix of ECM proteins that are deposited by fibroblasts. FDMs, while they provide a relevant tumour microenvironment, are easily manipulated, which limits their versatility. Furthermore, fibroblasts in culture change their phenotype (such as an increase in αSMA expression [42]) to varying degrees, which requires careful phenotypic and molecular monitoring. A good test of our drug screening platform would be to see whether a clinically effective kinase therapy was effective on our FDMs but not on other 2D substrates. This would demonstrate the further clinical relevance of FDMs. Here, we show that we can use FDMs to perform high-throughput drug screening and highlight that using an ECM substrate during this process is of critical importance.

In our drug screens, we treated 4T1 cells with kinase inhibitors at one concentration (1 μM) for a set time (3 days). The inhibitory capabilities of these inhibitors differ when 4T1 cells are on plastic or ECM. There are drugs that show efficacy when 4T1s are on plastic but not on ECM, irrespective of the matrix substrate (gelatin, Matrigel, or lung FDM), which we hypothesise have less clinical relevance, as they do not encompass the complex protein composition seen in vivo. We know the ECM can act as a barrier to drug delivery to cancer cells [43] and, as such, is a vital element in drug testing. The differences that we see in drug response to kinase inhibition are also validated as the global kinase signalling that we have performed clearly shows that activation of kinases differs greatly between 4T1 cells on ECM and plastic.

Here, we present a novel method of performing large-scale drug screening of kinase inhibitor, which encompasses the ECM by using FDMs. While we provide several ECM substrates that could be used to represent the ECM for drug screening, we observe that the FDMs contain the complex mixture of ECM proteins, in addition to other biophysical cues, that might best represent the in vivo situation. We also show that using matrices from different metastatic organs in breast cancer can elucidate subtle changes in kinase signalling pathways that could be targeted. Moreover, in this study, we highlight that there is a real need to incorporate the ECM into in vitro drug screening as results from screening on plastic alone do not replicate those of 4T1 cells on ECM. We therefore stress the importance of including ECM during in vitro breast cancer metastasis investigations, including drug screening.

3. Conclusions

The conclusions of this study are as follows:

- ECM produced by fibroblasts from different murine organs (to which breast cancer metastasise) is complex, abundant in ECM constituents, and differ in composition between organs.
- FDMs can be used to assess global kinase signalling of 4T1 and 67NR breast cancer cells in vitro. Inter-organ kinase activation differs to a smaller degree compared to cancer cells on plastic.
- FDMs can be used to perform high-throughput screening of kinase inhibitors on 4T1 cells. Inhibition of 4T1 proliferation on ECM differs from 4T1 cells screened on plastic.
- Network analysis performed proposes targets to inhibit proliferation of 4T1 cells in the lung by combining lung ECM changes with kinase activation profiles, and inferring protein–protein interaction pathways.

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